

09/24/99

JCS34 U.S. PTO

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**DIVISION - CONTINUATION - CONTINUATION-IN-PART  
APPLICATION TRANSMITTAL FORM**

Attorney Docket No.:  
A-378-CIP2C2

Anticipated Classification Of This Application:  
Class Subclass

Prior Application:  
Examiner  
Teng, S.

Art Unit  
1646

JCS34 U.S. PTO  
09/405032

09/24/99

To the Assistant Commissioner for Patents:

This is a request for filing a ☒ continuation ☐ divisional ☐ continuation-in-part application, under 37 CFR 1.53(b), of pending prior application Serial No. 09/132,985 filed on August 12, 19 98, of William J. Boyle, David L. Lacey, Frank J. Calzone and Ming-Shi Chang for OSTEOPROTEGERIN

For CONTINUATION or DIVISIONAL APPLNs only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 1b, below, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

1. ☒ Transmitted herewith are:  
☒ 138 pages of specification, 9 pages of claim(s) and 1 page of abstract, **totaling** 148 pages.  
☒ 46 sheet(s) of drawings.  
☒ 3 pages of Oath or Declaration by the applicant(s):  
☐ a. Newly executed (original or copy)  
☒ b. Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional applns. only)  
☒ 80 pages of Sequence Listing; computer readable copy (identical to paper copy); sequence statement.
2. ☒ The filing fee is calculated below:

For	Number Filed		Number Extra		Rate		Fee
Total Claims	8	- 20 =	0	x	\$18.00	=	\$ 0.00
Independent Claims	1	- 3 =	0	x	\$78.00	=	0.00
Multiple Dependent Claims	0			+	\$260.00	=	0.00
Basic Fee					\$760.00	=	760.00
Total Filing Fee							\$ 760.00

3. ☒ Please charge Deposit Account No. 01-0519, in the name of Amgen Inc., in the amount of \$ 760.00. An original and one copy are enclosed.
4. ☒ Throughout the prosecution of this application, if any extension of time is necessary, please consider this a request therefor.
5. ☒ The Commissioner is hereby authorized to charge any additional filing fees which may be required by the accompanying application, any additional fees which may be required during pendency of this application as required by 37 CFR 1.16 or 1.17, or credit any overpayment to Deposit Account No. 01-0519 throughout the prosecution of this application.
6. ☒ Cancel in this application original claims 1-60 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

**EXPRESS MAIL CERTIFICATE**

"Express Mail" mail labeling number EL198792556US Date of Deposit September 24, 1999  
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and addressed to Box Patent Application, Assistant Commissioner for Patents, Washington, DC 20231.  
RC. RASE Printed Name F. Craft Signature

7. ☐ Preliminarily, please amend the specification by inserting before the first line the following:

--This application is a ☒ continuation of Serial No. 08/771,777, filed 12/20/96, which is a continuation in part of 08/706,945, filed 9/3/96, which is a continuation in part of 08/577,788, filed 12/22/95 which is hereby incorporated by reference.--

8. ☐ Transfer the drawings from the prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file. (May only be used if signed by person authorized by § 1.138 and before payment of base issue fee.)

8a. ☐ New formal drawings are enclosed.

9. ☐ Priority of application Serial No. \_\_\_\_\_ filed on \_\_\_\_\_ in \_\_\_\_\_ (country) is claimed under 35 USC 119.

9a. ☐ The certified copy has been filed in prior application Serial No. \_\_\_\_\_ filed \_\_\_\_\_

10. ☒ The prior application is assigned of record to Amgen Inc.

11. ☒ A preliminary amendment is enclosed.

12. ☒ Also enclosed Attorney's Statement

13. ☐ Other: \_\_\_\_\_

14. ☒ The power of attorney in the prior application is to:  
Ron K. Levy, Registration No. 31,539; Steven M. Ode, Registration No. 29,094; and  
Robert B. Winter, Registration No. 34,458

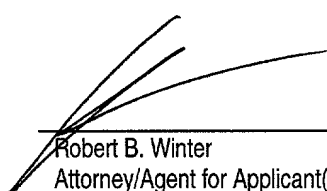
a. ☒ The power appears in the original papers in the prior application.

b. ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

c. ☒ Address all future communications to  
Robert B. Winter  
 at the address below.

Signator: ☐ Assignee of complete interest

☒ Attorney or agent of record

  
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 Date: September 24, 1999

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant(s): Boyle et al.

Serial No.: Continuation of 09/132,985

Filed: September 24, 1999

For: Osteoprotegerin

Docket No.: A-378-CIP2C2

**PRELIMINARY AMENDMENT**

This preliminary amendment accompanies the filing of a continuation application under 37 CFR 1.53(b).

**AMENDMENT**

Please amend the application as follows:

In the specification:

At p. 1, line 1, insert the following:

-- This application is a continuation of Serial No. 09/132,985 filed August 12, 1998, which is a continuation of Serial No. 08/771,777, filed December 20, 1996, which is a continuation in part of 08/706,945, filed September 3, 1996, which is a continuation in part of 08/577,788, filed December 22, 1995 which is hereby incorporated by reference --.

In the claims:

Please cancel Claims 1-60 without prejudice and add the following new claims.

-- 61. (new) A method for treating bone loss in a mammal comprising administering to the mammal an expression vector comprising a nucleic acid sequence encoding osteoprotegerin.

62. (new) The method of Claim 61 wherein the nucleic acid sequence is selected from the group consisting of:

a) a nucleic acid encoding a polypeptide comprising the amino acid sequence from residues 1 to 401 or from residues 22 to 401 as shown in Figure 9C-9D (SEQ ID NO:124);

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Signature

b) a nucleic acid encoding the polypeptide as in (a) wherein the polypeptide comprises from 1 to 216 amino acid residues deleted from the carboxy terminus; and  
c) a nucleic acid which hybridizes under high stringency conditions with the nucleic acid set forth in (a) and (b) wherein the hybridizing nucleic acid encodes a polypeptide having the activity of inhibiting bone resorption.

63. (new) The method of Claim 62 wherein the nucleic acid encodes a polypeptide comprising residues 22-185, 22-189, 22-194, or 22-201 inclusive as shown in Figure 9C-9D (SEQ ID NO:124).

64. (new) The method of Claim 62 wherein the nucleic acid further comprises an Fc region of human IgG.

65. (new) The method of Claim 61 wherein the expression vector is a viral vector.

66. (new) The method of Claim 61 wherein the expression vector further comprises a pharmaceutically acceptable adjuvant.

67. (new) The method of Claim 61 wherein the bone loss is due to osteoporosis.

68. (new) The method of Claim 61 wherein the bone loss is due to rheumatoid arthritis.

#### REMARKS

Applicants have canceled Claims 1-60 which are pending in the parent application, U.S. Serial No. 09/132,985 without prejudice and reserve the right to introduce claims relating to the subject matter therein in timely filed continuation applications.

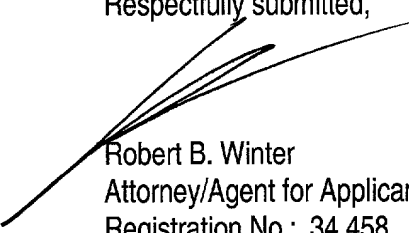
Claims 61-68 have been added which are directed to treatment of bone loss by administering an expression vector comprising a nucleic acid encoding osteoprotegerin. It is believed that the claims do not introduce new matter or new issues requiring search. Entry of the new claims is respectfully requested.



## CONCLUSION

It is submitted that Claims 61-68 are in condition for allowance and an early notice thereof is solicited.

Respectfully submitted,



Robert B. Winter  
Attorney/Agent for Applicant(s)  
Registration No.: 34,458  
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Please send all future correspondence to:

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## OSTEOPROTEGERIN

### Field of the Invention

5           ✓The invention relates generally to  
polypeptides involved in the regulation of bone  
metabolism. More particularly, the invention relates  
to a novel polypeptide, termed osteoprotegerin, which  
is a member of the tumor necrosis factor receptor  
superfamily. The polypeptide is used to treat bone  
10 diseases characterized by increased bone loss such as  
osteoporosis.

### Background of the Invention

15           Polypeptide growth factors and cytokines are  
secreted factors which signal a wide variety of changes  
in cell growth, differentiation, and metabolism, by  
specifically binding to discrete, surface bound  
receptors. As a class of proteins, receptors vary in  
their structure and mode of signal transduction. They  
20 are characterized by having an extracellular domain  
that is involved in ligand binding, and cytoplasmic  
domain which transmits an appropriate intracellular  
signal. Receptor expression patterns ultimately  
determine which cells will respond to a given ligand,  
25 while the structure of a given receptor dictates the  
cellular response induced by ligand binding. Receptors  
have been shown to transmit intracellular signals via  
their cytoplasmic domains by activating protein  
tyrosine, or protein serine/threonine phosphorylation  
30 (e.g., platelet derived growth factor receptor (PDGFR)  
or transforming growth factor- $\beta$  receptor-I (TGF $\beta$ R-I),  
by stimulating G-protein activation (e.g.,  $\beta$ -adrenergic  
receptor), and by modulating associations with

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Ellen Sorensen

Printed Name

*Ellen Sorensen*  
Signature

cytoplasmic signal transducing proteins (e.g., TNFR-1 and Fas/APO) (Heldin, Cell 80, 213-223 (1995)).

The tumor necrosis factor receptor (TNFR) superfamily is a group of type I transmembrane proteins which share a conserved cysteine-rich motif which is repeated three to six times in the extracellular domain (Smith, et al. Cell 76, 953-962 (1994)). Collectively, these repeat units form the ligand binding domains of these receptors (Chen et al., Chemistry 270, 2874-2878 (1995)). The ligands for these receptors are a structurally related group of proteins homologous to TNF $\alpha$ . (Goeddel et al. Cold Spring Harbor Symp. Quart. Biol. 51, 597-609 (1986); Nagata et al. Science 267, 1449-1456 (1995)). TNF $\alpha$  binds to distinct, but closely related receptors, TNFR-1 and TNFR-2. TNF $\alpha$  produces a variety of biological responses in receptor bearing cells, including, proliferation, differentiation, and cytotoxicity and apoptosis (Beutler et al. Ann. Rev. Biochem. 57, 505-518 (1988)).

TNF $\alpha$  is believed to mediate acute and chronic inflammatory responses (Beutler et al. Ann. Rev. Biochem. 57, 505-508 (1988)). Systemic delivery of TNF $\alpha$  induces toxic shock and widespread tissue necrosis. Because of this, TNF $\alpha$  may be responsible for the severe morbidity and mortality associated with a variety of infectious diseases, including sepsis. Mutations in FasL, the ligand for the TNFR-related receptor Fas/APO (Suda et al. Cell 75, 1169-1178 (1993)), is associated with autoimmunity (Fisher et al. Cell 81, 935-946 (1995)), while overproduction of FasL may be implicated in drug-induced hepatitis. Thus, ligands to the various TNFR-related proteins often mediate the serious effects of many disease states, which suggests that agents that neutralize the activity of these ligands would have therapeutic value. Soluble TNFR-1

receptors, and antibodies that bind TNF $\alpha$ , have been tested for their ability to neutralize systemic TNF $\alpha$  (Loetscher et al. Cancer Cells 3(6), 221-226 (1991)). A naturally occurring form of a secreted TNFR-1 mRNA was recently cloned, and its product tested for its ability to neutralize TNF $\alpha$  activity in vitro and in vivo (Kohn et al. PNAS USA 87, 8331-8335 (1990)). The ability of this protein to neutralize TNF $\alpha$  suggests that soluble TNF receptors function to bind and clear TNF thereby blocking the cytotoxic effects on TNFR-bearing cells.

An object of the invention to identify new members of the TNFR super family. It is anticipated that new family members may be transmembrane proteins or soluble forms thereof comprising extracellular domains and lacking transmembrane and cytoplasmic domains. We have identified a new member of the TNFR superfamily which encodes a secreted protein that is closely related to TNFR-2. By analogy to soluble TNFR-1, the TNFR-2 related protein may negatively regulate the activity of its ligand, and thus may be useful in the treatment of certain human diseases.

#### Summary of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily has been identified from a fetal rat intestinal cDNA library. A full-length cDNA clone was obtained and sequenced. Expression of the rat cDNA in a transgenic mouse revealed a marked increase in bones density, particularly in long bones, pelvic bone and vertebrae. The polypeptide encoded by the cDNA is termed Osteoprotegerin (OPG) and plays a role in promoting bone accumulation.

The invention provides for nucleic acids encoding a polypeptide having at least one of the

biological activities of OPG. Nucleic acids which hybridize to nucleic acids encoding mouse, rat or human OPG as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO: 122), and 9C-9D (SEQ ID NO: 124) are also provided. Preferably, OPG is mammalian OPG and more preferably is human OPG. Recombinant vectors and host cells expressing OPG are also encompassed as are methods of producing recombinant OPG. Antibodies or fragments thereof which specifically bind the polypeptide are also disclosed.

Methods of treating bone diseases are also provided by the invention. The polypeptides are useful for preventing bone resorption and may be used to treat any condition resulting in bone loss such as osteoporosis, hypercalcemia, Paget's disease of bone, and bone loss due to rheumatoid arthritis or osteomyelitis, and the like. Bone diseases may also be treated with anti-sense or gene therapy using nucleic acids of the invention. Pharmaceutical compositions comprising OPG nucleic acids and polypeptides are also encompassed.

#### Description of the Figures

Figure 1. A. FASTA analysis of novel EST LORF. Shown is the deduced FRI-1 amino acid sequence aligned to the human TNFR-2 sequence. B. Profile analysis of the novel EST LORF shown is the deduced FRI-1 amino acid sequence aligned to the TNFR-profile. C. Structural view of TNFR superfamily indicating region which is homologous to the novel FRI-1.

Figure 2. Structure and sequence of full length rat OPG gene, a novel member of the TNFR superfamily. A. Map of pMOB-B1.1 insert. Box indicates position of LORF within the cDNA sequence (bold line). Black box

indicates signal peptide, and gray ellipses indicate position of cysteine-rich repeat sequences. B, C. Nucleic acid and protein sequence of the Rat OPG cDNA. The predicted signal peptide is underlined, and  
5 potential sites of N-linked glycosylation are indicated in bold, underlined letters. D, E. Pileup sequence comparison (Wisconsin GCG Package, Version 8.1) of OPG with other members of the TNFR superfamily, fas (SEQ ID NO:128); tnfr1 (SEQ ID NO: 129); sfu-t2 (SEQ ID  
10 NO:130); tnfr2 (SEQ ID NO:131); cd40 (SEQ ID NO:132); osteo (SEQ ID NO:133); ngfr (SEQ ID NO:134); ox40 (SEQ ID NO:135); 41bb (SEQ ID NO:136).

Figure 3. PepPlot analysis (Wisconsin GCG Package,  
15 Version 8.1) of the predicted rat OPG protein sequence. A. Schematic representation of rat OPG showing hydrophobic (up) and hydrophilic (down) amino acids. Also shown are basic (up) and acidic (down) amino acids. B. Display of amino acid residues that are  
20 beta-sheet forming (up) and beta-sheet breaking down) as defined by Chou and Fasman (Adv. Enz. 47, 45-147 (1948)). C. Display of propensity measures for alpha-helix and beta-sheet (Chou and Fasman, ibid). Curves above 1.00 show propensity for alpha-helix or beta-  
25 sheet structure. Structure may terminate in regions of protein where curves drop below 1.00. D. Display of residues that are alpha-forming (up) or alpha-breaking (down). E. Display of portions of the protein sequence that resemble sequences typically found at the amino  
30 end of alpha and beta structures (Chou and Fasman, ibid). F. Display of portions of the protein sequence that resemble sequences typically found at the carboxyl end of alpha and beta structures (Chou and Fasman, ibid). G. Display of portions of the proteins sequence  
35 typically found in turns (Chou and Fasman, ibid) H. Display of the helical hydrophobic moment (Eisenberg et

al. Proc. Natl. Acad. Sci. USA 81, 140-144 (1984)) at  
each position in the sequence. I. Display of average  
hydrophathy based upon Kyte and Doolittle (J. Mol.  
Biol. 157, 105-132 (1982)) and Goldman et al. (reviewed  
5 in Ann. Rev. Biophys. Biophys. Chem. 15, 321-353  
(1986)).

Figure 4. mRNA expression patterns for the OPG cDNA in  
human tissues. Northern blots were probed with a 32P-  
10 labeled rat cDNA insert (A, left two panels), or with  
the human cDNA insert (B, right panel).

Figure 5. Creation of transgenic mice expressing the  
OPG cDNA in hepatocytes. Northern blot expression of  
15 HE-OPG transgene in mouse liver.

Figure 6. Increase in bone density in OPG transgenic  
mice. Panel A-F. Control Mice. G-J, OPG expressing  
mice. At necropsy, all animals were radiographed and  
20 photographs prepared. In A-F, the radiographs of the  
control animals and the one transgenic non-expressor  
(#28) are shown. Note that the bones have a clearly  
defined cortex and a lucent central marrow cavity. In  
contrast, the OPG (G-J) animals have a poorly defined  
25 cortex and increased density in the marrow zone.

Figure 7. Increase in trabecular bone in OPG  
transgenic mice. A-D. Representative photomicrographs  
of bones from control animals. In A and B, low (4X,  
30 10X) power images of the femurs are shown (Masson  
Trichrome stain). Stains for tartrate resistant acid  
phosphatase (TRAP) demonstrate osteoclasts (see arrows)  
both resorbing cartilage (C) and trabecular bone (D).  
Note the flattened appearance of osteoclasts on  
35 trabecular bone. E-H. Representative  
photomicrographs of bones from OPG-expressing animals.

In E and F, low (4X; 10X) power images of the femurs are shown (Masson Trichrome stain). The clear region is the growth plate cartilage, blue stained area is bone, and the red area is marrow. Note that in contrast to the controls, the trabecular bone has not been resorbed resulting in the absence of the usual marrow cavity. Also, the resulting trabeculae have a variegated appearance with blue and clear areas. The clear areas are remnants of growth plate cartilage that have never been remodelled. Based on TRAP stains, these animals do have osteoclasts (see arrows) at the growth plate (G), which may be reduced in number. However, the surfaces of the trabeculae away from the growth plate are virtually devoid of osteoclasts (H), a finding that stands in direct contrast with the control animals (see D).

Figure 8. HE-OPG expressors do not have a defect in monocyte-macrophage development. One cause for osteopetrosis in mice is defective M-CSF production due to a point mutation in the M-CSF gene. This results in a marked deficit of circulating and tissue based macrophages. The peripheral blood of OPG expressors contained monocytes as assessed by H1E analysis. To affirm the presence of tissue macrophages, immunohistochemistry was performed using F480 antibodies, which recognize a cell surface antigen on murine macrophages. A and C show low power (4X) photomicrographs of the spleens from normal and CR1 overexpressors. Note that both animals have numerous F480 positive cells. Monocyte-macrophages were also present in the marrow of normal (B) and HE-OPG overexpressors (D) (40X).

Figure 9. Structure and sequence of mouse and human OPG cDNA clones. A, B. Mouse cDNA and protein



sequence. C, D. Human cDNA and protein sequence. The predicted signal peptides are underlined, and potential sites of N-linked glycosylation are indicated in bold. E, F. Sequence alignment and comparison of rat, mouse and human OPG amino acid sequences.

Figure 10. Comparison of conserved sequences in extracellular domain of TNFR-1 and human OPG. PrettyPlot (Wisconsin GCG Package, Version 8.1) of the TNFR1 and OPG alignment described in example 6. Top line, human TNFR1 sequences encoding domains 1-4. Bottom line, human OPG sequences encoding domains 1-4. Conserved residues are highlighted by rectangular boxes.

Figure 11. Three-dimensional representation of human OPG. Side-view of the Molescript display of the predicted 3-dimensional structure of human OPG residues 25 through 163, (wide line), co-crystallized with human TNF $\beta$  (thin line). As a reference for orientation, the bold arrows along the OPG polypeptide backbone are pointing in the N-terminal to C-terminal direction. The location of individual cysteine residue side chains are inserted along the polypeptide backbone to help demonstrate the separate cysteine-rich domains. The TNF $\beta$  molecule is aligned as described by Banner et al. (1993).

Figure 12. Structure of OPG cysteine-rich domains. Alignment of the human (top line SEQ ID NO:136) and mouse (bottom line) OPG amino acid sequences highlighting the predicted domain structure of OPG. The polypeptide is divided into two halves; the N-terminus (A), and C-terminus (B). The N-terminal half is predicted to contain four cysteine rich domains (labeled 1-4). The predicted intrachain disulfide

bonds are indicated by bold lines, labeled "SS1",  
"SS2", or "SS3". Tyrosine 28 and histidine 75  
(underlined) are predicted to form an ionic  
interaction. Those amino acids predicted to interact  
5 with an OPG ligand are indicated by bold dots above the  
appropriate residue. The cysteine residues located in  
the C-terminal half of OPG are indicated by rectangular  
boxes.

10 Figure 13. Expression and secretion of full length and  
truncated mouse OPG-Fc fusion proteins. A. Map  
indicating points of fusion to the human IgG1 Fc domain  
are indicated by arrowheads. B. Silver stain of a  
SDS-polyacrylamide gel of conditioned media obtained  
15 from cells expressing either Fl.Fc (Full length OPG  
fused to Fc at Leucine 401) or CT.Fc (Carboxy-terminal  
truncated OPG fused to Fc at threonine 180) fusion  
protein expression vectors. Lane 1, parent pCEP4  
expression vector cell line; Lane 2, Fl.Fc vector cell  
20 line; Lane 3, CT.Fc vector cell line. C. Western  
blot of conditioned media obtained from Fl.Fc and CT.Fc  
fusion protein expression vectors probed with anti-  
human IgG1 Fc domain (Pierce). Lane 1, parent pCEP4  
expression vector cell line; Lane 2, Fl.Fc vector cell  
25 line; Lane 3, CT.Fc vector cell line.

Figure 14. Expression of human OPG in E. coli. A.  
Construction of a bacterial expression vector. The  
LORF of the human OPG gene was amplified by PCR, then  
30 joined to a oligonucleotide linker fragment (top strand  
is SEQ ID NO:137; bottom strand is SEQ ID NO:127), and  
ligated into pAMG21 vector DNA. The resulting vector  
is capable of expressing OPG residues 32-401 linked to  
a N-terminal methionine residue. B SDS-PAGE analysis  
35 of uninduced and induced bacterial harboring the  
pAMG21-human OPG -32-401 plasmid. Lane 1, MW

standards; lane 2, uninduced bacteria; lane 3, 30°C induction; lane 4, 37°C induction; lane 5, whole cell lysate from 37°C induction; lane 6, soluble fraction of whole cell lysate; lane 7, insoluble fraction of whole cell lysate; lane 8, purified inclusion bodies obtained from whole cell lysate.

Figure 15. Analysis of recombinant murine OPG produced in CHO cells by SDS-PAGE and western blotting. An equal amount of CHO conditioned media was applied to each lane shown, and was prepared by treatment with either reducing sample buffer (left lane), or non-reducing sample buffer (right lane). After electrophoresis, the resolved proteins were transferred to a nylon membrane, then probed with anti-OPG antibodies. The relative positions of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 16. Pulse-chase analysis of recombinant murine OPG produced in CHO cells. CHO cells were pulse-labeled with <sup>35</sup>S-methionine/cysteine, then chased for the indicated time. Metabolically labeled cultures were separated into both conditioned media and cells, and detergent extracts were prepared from each, clarified, then immunoprecipitated with anti-OPG antibodies. The immunoprecipitates were resolved by SDS-PAGE, and exposed to film. Top left and right panels; samples analyzed under non-reducing conditions. Lower left and right panels; samples analyzed under reducing conditions. Top and bottom left panels; Cell extracts. Top and bottom right panels; Conditioned media extracts. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 17. Expression of OPG in the CTLL-2 cell line. Serum-free conditioned media from CTLL-2 cells and CHO-mu OPG [1-401] transfected cells was prepared, concentrated, then analyzed by non-reducing SDS-PAGE and western blotting. Left lane; CTLL-2 conditioned media. Right lane; CHO-muOPG conditioned media. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

Figure 18. Detection of OPG expression in serum samples and liver extracts obtained from control and OPG transgenic mice. Transgenic mice were constructed as described in Example 4. OPG expression was visualized after SDS-PAGE followed by Western blotting using anti-OPG antibodies.

Figure 19. Effects of huOPG [22-401]-Fc fusion protein on osteoclast formation in vitro. The osteoclast forming assay was performed as described in Example 11A in the absence (control) or presence of the indicated amounts of huOPG [22-401]-Fc fusion. Osteoclast formation was visualized by histochemical staining for tartrate acid phosphatase (TRAP). ). A. OPG added to 100 ng/ml. D. OPG added to 0.1 ng/ml. E. OPG added to 0.01 ng/ml. F. OPG added to 0.001 ng/ml. G. Control. No OPG added.

Figure 20. Decrease in osteoclast culture TRAP activity with increasing amounts of OPG. Indicated concentrations of huOPG [22-401]-Fc fusion protein were added to osteoclast forming assay and TRAP activity quantitated as described in Example 11A.

Figure 21. Effect of OPG on a terminal stage of osteoclast differentiation. huOPG [22-401]-Fc fusion was added to the osteoclast forming assay during the

intermediate stage of osteoclast maturation (days 5-6; OPG-CTL) or during the terminal stage of osteoclast maturation (days 7-15; CTL-OPG). TRAP activity was quantitated and compared with the activity observed in the absence of OPG (CTL-CTL) in the presence of OPG throughout (OPG-OPG).

Figure 22. Effects of IL-1 $\beta$ , IL-1 $\alpha$  and OPG on blood ionized calcium in mice. Levels of blood ionized calcium were monitored after injection of IL-1 $\beta$  alone, IL-1 $\alpha$  alone, IL-1 $\beta$  plus muOPG [22-401]-Fc, IL-1 $\alpha$  plus MuOPG [22-401]-Fc, and muOPG [22-401]-Fc alone. Control mice received injections of phosphate buffered saline (PBS) only. IL-1 $\beta$  experiment shown in A; IL-1 $\alpha$  experiment shown in B.

Figure 23. Effects of OPG on calvarial osteoclasts in control and IL1-treated mice. Histological methods for analyzing mice calvarial bone samples are described in Example 11B. Arrows indicate osteoclasts present in day 2-treated mice. Calvarial samples of mice receiving four PBS injections daily (A), one injection of IL-1 and three injections of PBS daily (B), one injection of PBS and three injections of OPG daily (C), one injection of IL-1 and three injections of OPG daily.

Figure 24. Radiographic analysis of bone accumulation in marrow cavity of normal mice. Mice were injected subcutaneously with saline (A) or muOPG [22-401]-Fc fusion (5mg/kg/d) for 14 days (B) and bone density determined as described in Example 11C.

Figure 25. Histomorphometric analysis of bone accumulation in marrow cavity of normal mice.

Injection experiments and bone histology performed as described in Example 11C.

Figure 26. Histology analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 11C. A. Saline injection B. Injection of muOPG [22-401]-Fc fusion.

Figure 27. Activity of OPG administered to ovariectomized rats. In this two week experiment the trend to reduced bone density appears to be blocked by OPG or other anti-resorptive therapies. DEXA measurements were taken at time of ovariectomy and at week 1 and week 2 of treatment. The results are expressed as % change from the initial bone density (Mean +/- SEM).

Figure 28. Bone density in the femoral metaphysis, measured by histomorphometric methods, tends to be lower in ovariectomized rats (OVX) than sham operated animals (SHAM) 17 days following ovariectomy. This effect was blocked by OPG-Fc, with OPG-Fc treated ovariectomized rats (OVX+OPG) having significantly higher bone density than vehicle treated ovariectomized rats (OVX). (Mean +/- SEM).

#### Detailed Description of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily was identified as an expressed sequence tag (EST) isolated from a fetal rat intestinal cDNA library. The structures of the full-length rat cDNA clones and the corresponding mouse and human cDNA clones were determined as described in Examples 1 and 6. The rat, mouse and human genes are

shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), respectively. All three sequences showed strong similarity to the extracellular domains of TNFR family members. None of the full-length cDNA clones isolated encoded transmembrane and cytoplasmic domains that would be expected for membrane-bound receptors, suggesting that these cDNAs encode soluble, secreted proteins rather than cell surface receptors. A portion of the human gene spanning nucleotides 1200-1353 shown in Figure 9D was deposited in the Genebank database on November 22, 1995 under accession no. 17188769.

The tissue distribution of the rat and human mRNA was determined as described in Example 2. In rat, mRNA expression was detected in kidney, liver, placenta and heart with the highest expression in the kidney. Expression in skeletal muscle and pancreas was also detected. In humans, expression was detected in the same tissues along with lymph node, thymus, spleen and appendix.

The rat cDNA was expressed in transgenic mice (Example 3) using the liver-specific ApoE promoter expression system. Analysis of expressors showed a marked increase in bone density, particularly in long bones (femurs), vertebrae and flat bones (pelvis). Histological analysis of stained sections of bone showed severe osteopetrosis (see Example 4) indicating a marked imbalance between bone formation and resorption which has led to a marked accumulation of bone and cartilage. A decrease in the number of trabecular osteoclasts in the bones of OPG expressor animals indicate that a significant portion of the activity of the TNFR-related protein may be to prevent bone resorption, a process mediated by osteoclasts. In view of the activity in transgenic expressors, the TNFR-related proteins described herein are termed OPGs.

Using the rat cDNA sequence, mouse and human cDNA clones were isolated (Example 5). Expression of mouse OPG in 293 cells and human OPG in E. coli is described in Examples 7 and 8. Mouse OPG was produced as an Fc fusion which was purified by Protein A affinity chromatography. Also described in Example 7 is the expression of full-length and truncated human and mouse OPG polypeptides in CHO and 293 cells either as fusion polypeptides to the Fc region of human IgG1 or as unfused polypeptides. The expression of full-length and truncated human and mouse OPGs in E. coli either as Fc fusion polypeptides or as unfused polypeptides is described in Example 8. Purification of recombinantly produced mammalian and bacterial OPG is described in Example 10.

The biological activity of OPG was determined using an in vitro osteoclast maturation assay, an in vivo model of interleukin-1 (IL-1) induced hypercalcemia, and injection studies of bone density in normal mice (see Example 11). The following OPG recombinant proteins produced in CHO or 293 cells demonstrated activity in the in E. coli osteoclast maturation assay: muOPG [22-185]-Fc, muOPG [22-194]-Fc, muOPG [22-401]Fc, muOPG [22-401], huOPG [22-201]-Fc, huOPG [22-401]-Fc. muOPG [22-180]-Fc produced in CHO cells and huOPG met[32-401] produced in E. coli did not demonstrate activity in the in vitro assay.

OPG from several sources was produced as a dimer and to some extent as a higher multimer. Rat OPG [22-401] produced in transgenic mice, muOPG [22-401] and huOPG [22-401] produced as a recombinant polypeptide in CHO cells, and OPG expressed as a naturally occurring product from a cytotoxic T cell line were predominantly dimers and trimers when analyzed on nonreducing SDS gels (see Example 9). Truncated OPG polypeptides having deletions in the



region of amino acids 186-401 (e.g., OPG [1-185] and  
OPG [1-194]) were predominantly monomeric suggesting  
that the region 186-401 may be involved in self-  
association of OPG polypeptides. However, huOPG  
5 met[32-401] produced in E. coli was largely monomeric.

OPG may be important in regulating bone  
resorption. The protein appears to act as a soluble  
receptor of the TNF family and may prevent a receptor-  
ligand interaction involved in the osteolytic pathway.  
10 One aspect of the regulation appears to be a reduction  
in the number of osteoclasts.

#### Nucleic Acids

The invention provides for an isolated  
15 nucleic acid encoding a polypeptide having at least one  
of the biological activities of OPG. As described  
herein, the biological activities of OPG include, but  
are not limited to, any activity involving bone  
metabolism and in particular, include increasing bone  
20 density. The nucleic acids of the invention are  
selected from the following:

- a) the nucleic acid sequences as shown in Figures  
2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D  
(SEQ ID NO:124) or complementary strands thereof;
- 25 b) the nucleic acids which hybridize under  
stringent conditions with the polypeptide-encoding  
region in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID  
NO:122), and 9C-9D (SEQ ID NO:124); and
- c) nucleic acids which hybridize under stringent  
30 conditions with nucleotides 148 through 337 inclusive  
as shown in Figure 1A.
- d) the nucleic acid sequences which are degenerate  
to the sequences in (a) and (b).

The invention provides for nucleic acids  
35 which encode rat, mouse and human OPG as well as  
nucleic acid sequences hybridizing thereto which encode

a polypeptide having at least one of the biological activities of OPG. Also provided for are nucleic acids which hybridize to a rat OPG EST encompassing nucleotides 148-337 as shown in Figure 1A. The conditions for hybridization are generally of high stringency such as 5xSSC, 50% formamide and 42°C described in Example 1 of the specification. Equivalent stringency to these conditions may be readily obtained by adjusting salt and organic solvent concentrations and temperature. The nucleic acids in (b) encompass sequences encoding OPG-related polypeptides which do not undergo detectable hybridization with other known members of the TNF receptor superfamily. In a preferred embodiment, the nucleic acids are as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124).

The length of hybridizing nucleic acids of the invention may be variable since hybridization may occur in part or all of the polypeptide-encoding regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), and may also occur in adjacent noncoding regions. Therefore, hybridizing nucleic acids may be truncations or extensions of the sequences shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124). Truncated or extended nucleic acids are encompassed by the invention provided they retain one or more of the biological properties of OPG. The hybridizing nucleic acids may also include adjacent noncoding regions which are 5' and/or 3' to the OPG coding region. The noncoding regions include regulatory regions involved in OPG expression, such as promoters, enhance, translational initiation sites, transcription termination sites and the like.

Hybridization conditions for nucleic acids are described in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)

5 DNA encoding rat OPG was provided in plasmid pMO-B1.1 deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under ATCC accession no. 69970. DNA encoding mouse OPG was provided in plasmid pRcCMV-murine OPG deposited with  
10 the American Type Culture Collection, Rockville, MD on December 27, 1995 under accession no. 69971. DNA encoding human OPG was provided in plasmid pRcCMV - human OPG deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under  
15 accession no. 69969. The nucleic acids of the invention will hybridize under stringent conditions to the DNA inserts of ATCC accession nos. 69969, 69970, and 69971 and have at least one of the biological activities of OPG.

20 Also provided by the invention are derivatives of the nucleic acid sequences as shown in Figures 2B, 9A and 9B. As used herein, derivatives include nucleic acid sequences having addition, substitution, insertion or deletion of one or more  
25 residues such that the resulting sequences encode polypeptides having one or more amino acid residues which have been added, deleted, inserted or substituted and the resulting polypeptide has the activity of OPG. The nucleic acid derivatives may be naturally  
30 occurring, such as by splice variation or polymorphism, or may be constructed using site-directed mutagenesis techniques available to the skilled worker. One example of a naturally occurring variant of OPG is a nucleic acid encoding a lys to asn change at residue 3  
35 within the leader sequence (see Example 5). It is anticipated that nucleic acid derivatives will encode

amino acid changes in regions of the molecule which are least likely to disrupt biological activity. Other derivatives include a nucleic acid encoding a membrane-bound form of OPG having an extracellular domain as  
5 shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) along with transmembrane and cytoplasmic domains.

In one embodiment, derivatives of OPG include nucleic acids encoding truncated forms of OPG having  
10 one or more amino acids deleted from the carboxy terminus. Nucleic acids encoding OPG may have from 1 to 216 amino acids deleted from the carboxy terminus. Optionally, an antibody Fc region may extend from the new carboxy terminus to yield a biologically active  
15 OPG-Fc fusion polypeptide. (see Example 11). In preferred embodiments, nucleic acids encode OPG having the amino acid sequence from residues 22-185, 22-189, 22-194 or 22-201 (using numbering in Figure 9E-F) and optionally, encoding an Fc region of human IgG.

Also included are nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the amino terminus. Truncated forms include those lacking part or all the 21 amino acids comprising the leader sequence. Additionally, the  
20 invention provides for nucleic acids encoding OPG having from 1 to 10 amino acids deleted from the mature amino terminus (at residue 22) and ,optionally, having from 1 to 216 amino acids deleted from the carboxy terminus (at residue 401). Optionally, the nucleic  
25 acids may encode a methionine residue at the amino terminus. Examples of such OPG truncated polypeptides are described in Example 8.

Examples of the nucleic acids of the invention include cDNA, genomic DNA, synthetic DNA and  
35 RNA. cDNA is obtained from libraries prepared from mRNA isolated from various tissues expressing OPG. In

humans, tissue sources for OPG include kidney, liver, placenta and heart. Genomic DNA encoding OPG is obtained from genomic libraries which are commercially available from a variety of species. Synthetic DNA is  
5 obtained by chemical synthesis of overlapping oligonucleotide fragments followed by assembly of the fragments to reconstitute part or all of the coding region and flanking sequences (see U.S. Patent No. 4,695,623 describing the chemical synthesis of  
10 interferon genes). RNA is obtained most easily by procaryotic expression vectors which direct high-level synthesis of mRNA, such as vectors using T7 promoters and RNA polymerase.

Nucleic acid sequences of the invention are  
15 used for the detection of OPG sequences in biological samples in order to determine which cells and tissues are expressing OPG mRNA. The sequences may also be used to screen cDNA and genomic libraries for sequences related to OPG. Such screening is well within the  
20 capabilities of one skilled in the art using appropriate hybridization conditions to detect homologous sequences. The nucleic acids are also useful for modulating the expression of OPG levels by anti-sense therapy or gene therapy. The nucleic acids are  
25 also used for the development of transgenic animals which may be used for the production of the polypeptide and for the study of biological activity (see Example 3).

### 30 Vectors and Host Cells

Expression vectors containing nucleic acid sequences encoding OPG, host cells transformed with said vectors and methods for the production of OPG are also provided by the invention. An overview of  
35 expression of recombinant proteins is found in Methods

of Enzymology v. 185, Goeddel, D.V. ed. Academic Press (1990).

Host cells for the production of OPG include procaryotic host cells, such as E. coli, yeast, plant, insect and mammalian host cells. E. coli strains such as HB101 or JM101 are suitable for expression. Preferred mammalian host cells include COS, CHOd-, 293, CV-1, 3T3, baby hamster kidney (BHK) cells and others. Mammalian host cells are preferred when post-translational modifications, such as glycosylation and polypeptide processing, are important for OPG activity. Mammalian expression allows for the production of secreted polypeptides which may be recovered from the growth medium.

Vectors for the expression of OPG contain at a minimum sequences required for vector propagation and for expression of the cloned insert. These sequences include a replication origin, selection marker, promoter, ribosome binding site, enhancer sequences, RNA splice sites and transcription termination site. Vectors suitable for expression in the aforementioned host cells are readily available and the nucleic acids of the invention are inserted into the vectors using standard recombinant DNA techniques. Vectors for tissue-specific expression of OPG are also included. Such vectors include promoters which function specifically in liver, kidney or other organs for production in mice, and viral vectors for the expression of OPG in targeted human cells.

Using an appropriate host-vector system, OPG is produced recombinantly by culturing a host cell transformed with an expression vector containing nucleic acid sequences encoding OPG under conditions such that OPG is produced, and isolating the product of expression. OPG is produced in the supernatant of transfected mammalian cells or in inclusion bodies of

transformed bacterial host cells. OPG so produced may be purified by procedures known to one skilled in the art as described below. The expression of OPG in mammalian and bacterial host systems is described in Examples 7 and 8. Expression vectors for mammalian hosts are exemplified by plasmids such as pDSR $\alpha$  described in PCT Application No. 90/14363. Expression vectors for bacterial host cells are exemplified by plasmids pAMG21 and pAMG22-His described in Example 8. Plasmid pAMG21 was deposited with the American Type Culture Collection, Rockville, MD on July 24, 1996 under accession no. 98113. Plasmid pAMG22-His was deposited with the American Type Culture Collection, Rockville, MD on July 24, 1996 under accession no. 98112. It is anticipated that the specific plasmids and host cells described are for illustrative purposes and that other available plasmids and host cells could also be used to express the polypeptides.

The invention also provides for expression of OPG from endogenous nucleic acids by in vivo or ex vivo recombination events to allow modulation of OPG from the host chromosome. Expression of OPG by the introduction of exogenous regulatory sequences (e.g. promoters or enhancers) capable of directing the production of OPG from endogenous OPG coding regions is also encompassed. Stimulation of endogenous regulatory sequences capable of directing OPG production (e.g. by exposure to transcriptional enhancing factors) is also provided by the invention.

#### Polypeptides

The invention provides for OPG, a novel member of the TNF receptor superfamily, having an activity associated with bone metabolism and in particular having the activity of inhibiting bone resorption thereby increasing bone density. OPG refers

to a polypeptide having an amino acid sequence of mouse, rat or human OPG or a derivative thereof having at least one of the biological activities of OPG. The amino acid sequences of rat, mouse and human OPG are shown in Figures 2B-2C (SEQ ID NO:121), 9A-9B (SEQ ID NO:123), and 9C-9D (SEQ ID NO:125) respectively. A derivative of OPG refers to a polypeptide having an addition, deletion, insertion or substitution of one or more amino acids such that the resulting polypeptide has at least one of the biological activities of OPG. The biological activities of OPG include, but are not limited to, activities involving bone metabolism. Preferably, the polypeptides will have the amino terminal leader sequence of 21 amino acids removed.

OPG polypeptides encompassed by the invention include rat [1-401], rat [22-180], rat [22-401], rat [22-401]-Fc fusion, rat [1-180]-Fc fusion, mouse [1-401], mouse [1-180], mouse [22-401], human [1-401], mouse [22-180], human [22-401], human [22-180], human [1-180], human [22-180]-Fc fusion and human met-32-401. Amino acid numbering is as shown in SEQ ID NO:121 (rat), SEQ ID NO:123 (mouse) and SEQ ID NO:125 (human). Also encompassed are polypeptide derivatives having deletions or carboxy-terminal truncations of part or all of amino acids residues 180-401 of OPG; one or more amino acid changes in residues 180-401; deletion of part or all of a cysteine-rich domain of OPG, in particular deletion of the distal (carboxy-terminal) cysteine-rich domain; and one or more amino acid changes in a cysteine-rich domain, in particular in the distal (carboxy-terminal) cysteine-rich domain. In one embodiment, OPG has from 1 to about 216 amino acids deleted from the carboxy terminus. In another embodiment, OPG has from 1 to about 10 amino acids deleted from the mature amino terminus (wherein the mature amino terminus is at residue 22) and,



optionally, has from 1 to about 216 amino acids deleted from the carboxy terminus.

Additional OPG polypeptides encompassed by the invention include the following: human [22-180]-Fc fusion, human [22-201]-Fc fusion, human [22-401]-Fc fusion, mouse [22-185]-Fc fusion, mouse [22-194]-Fc fusion. These polypeptides are produced in mammalian host cells, such as CHO or 293 cells, Additional OPG polypeptides encompassed by the invention which are expressed in procaryotic host cells include the following: human met[22-401], Fc-human met[22-401] fusion (Fc region is fused at the amino terminus of the full-length OPG coding sequence as described in Example 8), human met[22-401]-Fc fusion (Fc region fused to the full-length OPG sequence), Fc-mouse met[22-401] fusion, mouse met[22-401]-Fc fusion, human met[27-401], human met[22-185], human met[22-189], human met[22-194], human met[22-194] (P25A), human met [22-194] (P26A), human met[27-185], human met[27-189], human met[27-194], human met-arg-gly-ser-(his)<sub>6</sub> [22-401], human met-lys [22-401], human met-(lys)<sub>3</sub>-[22-401], human met[22-401]-Fc (P25A), human met[22-401] (P25A), human met[22-401] (P26A), human met[22-401] (P26D), mouse met[22-401], mouse met[27-401], mouse met[32-401], mouse met[27-180], mouse met[22-189], mouse met[22-194], mouse met[27-189], mouse met[27-194], mouse met-lys[22-401], mouse HEK[22-401] (A45T), mouse met-lys-(his)<sub>7</sub>[22-401], mouse met-lys[22-401]-(his)<sub>7</sub> and mouse met[27-401] (P33E, G36S, A45P). It is understood that the above OPG polypeptides produced in procaryotic host cells have an amino-terminal methionine residue, if such a residue is not indicated. In specific examples, OPG-Fc fusion were produced using a 227 amino acid region of human IgG1-γ1 was used having the sequence as shown in Ellison et al. (Nuc. Acids Res.

10, 4071-4079 (1982)). However, variants of the Fc region of human IgG may also be used.

Analysis of the biological activity of carboxy-terminal OPG truncations fused to the human IgG1 Fc region indicates a portion of OPG of about 164 amino acids which is required for activity. This region encompasses amino acids 22-185, preferably those in Figure 9C-9D (SEQ ID NO:125), and comprises four cysteine-rich domains characteristic of the cysteine-rich domains of TNFR extracellular domains.

Using the homology between OPG and the extracellular ligand binding domains of TNF receptor family members, a three-dimensional model of OPG was generated based upon the known crystal structure of the extracellular domain of TNFR-I (see Example 6). This model was used to identify those residues within OPG which may be important for biological activity. Cysteine residues that are involved in maintaining the structure of the four cysteine-rich domains were identified. The following disulfide bonds were identified in the model: Domain 1: cys41 to cys54, cys44 to cys62, tyr23 and his 66 may act to stabilize the structure of this domain; Domain 2: cys65 to cys80, cys83 to cys98, cys87 to cys105; Domain 3: cys107 to cys118, cys124 to cys142; Domain 4: cys145 to cys160, cys166 to cys185. Residues were also identified which were in close proximity to TNF $\beta$  as shown in Figures 11 and 12A-12B. In this model, it is assumed that OPG binds to a corresponding ligand; TNF $\beta$  was used as a model ligand to simulate the interaction of OPG with its ligand. Based upon this modeling, the following residues in OPG may be important for ligand binding: glu34, lys43, pro66 to gln91 (in particular, pro66, his68, tyr69, tyr70, thr71, asp72, ser73, his76, ser77, asp78, glu79, leu81, tyr82, pro85, val86, lys88, glu90 and gln91), glu153 and ser155.

Alterations in these amino acid residues, either singly or in combination, may alter the biological activity of OPG. For example, changes in specific cysteine residues may alter the structure of individual cysteine-rich domains, whereas changes in residues important for ligand binding may affect physical interactions of OPG with ligand. Structural models can aid in identifying analogs which have more desirable properties, such as enhanced biological activity, greater stability, or greater ease of formulation.

The invention also provides for an OPG multimer comprising OPG monomers. OPG appears to be active as a multimer (e.g, dimer, trimer of a higher number of monomers). Preferably, OPG multimers are dimers or trimers. OPG multimers may comprise monomers having the amino acid sequence of OPG sufficient to promote multimer formation or may comprise monomers having heterologous sequences such as an antibody Fc region. Analysis of carboxy-terminal deletions of OPG suggest that at least a portion of the region 186-401 is involved in association of OPG polypeptides. Substitution of part or all of the region of OPG amino acids 186-401 with an amino acid sequence capable of self-association is also encompassed by the invention. Alternatively, OPG polypeptides or derivatives thereof may be modified to form dimers or multimers by site directed mutagenesis to create unpaired cysteine residues for interchain disulfide bond formation, by photochemical crosslinking, such as exposure to ultraviolet light, or by chemical crosslinking with bifunctional linker molecules such as bifunctional polyethylene glycol and the like.

Modifications of OPG polypeptides are encompassed by the invention and include post-translational modifications (e.g., N-linked or O-linked

carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of  
5 an N-terminal methionine residue as a result of procaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

10 Further modifications of OPG include chimeric proteins wherein OPG is fused to a heterologous amino acid sequence. The heterologous sequence may be any sequence which allows the resulting fusion protein to retain the activity of OPG. The heterologous sequences  
15 include for example, immunoglobulin fusions, such as Fc fusions, which may aid in purification of the protein. A heterologous sequence which promotes association of OPG monomers to form dimers, trimers and other higher multimeric forms is preferred.

20 The polypeptides of the invention are isolated and purified from other polypeptides present in tissues, cell lines and transformed host cells expressing OPG, or purified from components in cell cultures containing the secreted protein. In one  
25 embodiment, the polypeptide is free from association with other human proteins, such as the expression product of a bacterial host cell.

Also provided by the invention are chemically modified derivatives of OPG which may provide  
30 additional advantages such as increasing stability and circulating time of the polypeptide, or decreasing immunogenicity (see U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene  
35 glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and

the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

5           The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1kDa and about 100kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules  
10 will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity,  
15 the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

          The polyethylene glycol molecules (or other  
20 chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, e.g. EP 0 401 384 herein incorporated by reference  
25 (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol. 20: 1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a  
30 free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free  
35 carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid

residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

One may specifically desire N-terminally chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemically modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

Synthetic OPG dimers may be prepared by various chemical crosslinking procedures. OPG monomers may be chemically linked in any fashion that retains or enhances the biological activity of OPG. A variety of chemical crosslinkers may be used depending upon which properties of the protein dimer are desired. For example, crosslinkers may be short and relatively rigid

or longer and more flexible, may be biologically reversible, and may provide reduced immunogenicity or longer pharmacokinetic half-life.

In one example, OPG molecules are linked through the amino terminus by a two step synthesis (see Example 12). In the first step, OPG is chemically modified at the amino terminus to introduce a protected thiol, which after purification is deprotected and used as a point of attachment for site-specific conjugation through a variety of crosslinkers with a second OPG molecule. Amino-terminal crosslinks include, but are not limited to, a disulfide bond, thioether linkages using short-chain, bis-functional aliphatic crosslinkers, and thioether linkages to variable length, bifunctional polyethylene glycol crosslinkers (PEG "dumbbells"). Also encompassed by PEG dumbbell synthesis of OPG dimers is a byproduct of such synthesis, termed a "monobell". An OPG monobell consists of a monomer coupled to a linear bifunctional PEG with a free polymer terminus. Alternatively, OPG may be crosslinked directly through a variety of amine specific homobifunctional crosslinking techniques which include reagents such as: diethylenetriaminepentaacetic dianhydride (DTPA), p-benzoquinone (pBQ) or bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>) as well as others known in the art. It is also possible to thiolate OPG directly with reagents such as iminothiolane in the presence of a variety of bifunctional, thiol specific crosslinkers, such as PEG bismaleimide, and achieve dimerization and/or dumbbells in a one step process.

A method for the purification of OPG from natural sources and from transfected host cells is also included. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The chromatography steps can include ion exchange, gel

filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-OPG antibody or biotin-streptavidin affinity complex and the like.

5

#### Antibodies

Also encompassed by the invention are antibodies specifically binding to OPG. Antigens for the generation of antibodies may be full-length polypeptides or peptides spanning a portion of the OPG sequence. Immunological procedures for the generation of polyclonal or monoclonal antibodies reactive with OPG are known to one skilled in the art (see, for example, Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y. (1988)). Antibodies so produced are characterized for binding specificity and epitope recognition using standard enzyme-linked immunosorbent assays. Antibodies also include chimeric antibodies having variable and constant domain regions derived from different species. In one embodiment, the chimeric antibodies are humanized antibodies having murine variable domains and human constant domains. Also encompassed are complementary determining regions grafted to a human framework (so-called CDR-grafted antibodies). Chimeric and CDR-grafted antibodies are made by recombinant methods known to one skilled in the art. Also encompassed are human antibodies made in mice.

Anti-OPG antibodies of the invention may be used as an affinity reagent to purify OPG from biological samples (see Example 10). In one method, the antibody is immobilized on CnBr-activated Sepharose and a column of antibody-Sepharose conjugate is used to remove OPG from liquid samples. Antibodies are also



used as diagnostic reagents to detect and quantitate OPG in biological samples by methods described below.

Pharmaceutical compositions

5                   The invention also provides for  
pharmaceutical compositions comprising a  
therapeutically effective amount of the polypeptide of  
the invention together with a pharmaceutically  
acceptable diluent, carrier, solubilizer, emulsifier,  
10 preservative and/or adjuvant. The term  
"therapeutically effective amount" means an amount  
which provides a therapeutic effect for a specified  
condition and route of administration. The composition  
may be in a liquid or lyophilized form and comprises a  
15 diluent (Tris, acetate or phosphate buffers) having  
various pH values and ionic strengths, solubilizer such  
as Tween or Polysorbate, carriers such as human serum  
albumin or gelatin, preservatives such as thimerosal or  
benzyl alcohol, and antioxidants such as ascorbic acid  
20 or sodium metabisulfite. Also encompassed are  
compositions comprising OPG modified with water soluble  
polymers to increase solubility or stability.  
Compositions may also comprise incorporation of OPG  
into liposomes, microemulsions, micelles or vesicles  
25 for controlled delivery over an extended period of  
time.  
Specifically, OPG compositions may comprise  
incorporation into polymer matrices such as hydrogels,  
silicones, polyethylenes, ethylene-vinyl acetate  
30 copolymers, or biodegradable polymers. Examples of  
hydrogels include polyhydroxyalkylmethacrylates (p-  
HEMA), polyacrylamide, polymethacrylamide,  
polyvinylpyrrolidone, polyvinyl alcohol and various  
polyelectrolyte complexes. Examples of biodegradable  
35 polymers include polylactic acid (PLA), polyglycolic  
acid (PGA), copolymers of PLA and PGA, polyamides and

copolymers of polyamides and polyesters. Other controlled release formulations include microcapsules, microspheres, macromolecular complexes and polymeric beads which may be administered by injection.

5        Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical  
10        compositions is found in Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1980).

             Compositions of the invention may be administered by injection, either subcutaneous,  
15        intravenous or intramuscular, or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one skilled in the art.

20        The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be  
25        suitable for the delivery of part or all of the OPG coding region to cells and tissues as part of an anti-sense or gene therapy regimen.

#### Methods of Treatment

30        Bone tissue provides support for the body and consists of mineral (largely calcium and phosphorous), a matrix of collagenous and noncollagenous proteins, and cells. Three types of cells found in bone, osteocytes, osteoblasts and osteoclasts, are involved  
35        in the dynamic process by which bone is continually formed and resorbed. Osteoblasts promote formation of

bone tissue whereas osteoclasts are associated with resorption. Resorption, or the dissolution of bone matrix and mineral, is a fast and efficient process compared to bone formation and can release large amounts of mineral from bone. Osteoclasts are involved in the regulation of the normal remodeling of skeletal tissue and in resorption induced by hormones. For instance, resorption is stimulated by the secretion of parathyroid hormone in response to decreasing concentrations of calcium ion in extracellular fluids. In contrast, inhibition of resorption is the principal function of calcitonin. In addition, metabolites of vitamin D alter the responsiveness of bone to parathyroid hormone and calcitonin.

After skeletal maturity, the amount of bone in the skeleton reflects the balance (or imbalance) of bone formation and bone resorption. Peak bone mass occurs after skeletal maturity prior to the fourth decade. Between the fourth and fifth decades, the equilibrium shifts and bone resorption dominates. The inevitable decrease in bone mass with advancing years starts earlier in females than males and is distinctly accelerated after menopause in some females (principally those of Caucasian and Asian descent).

Osteopenia is a condition relating generally to any decrease in bone mass to below normal levels. Such a condition may arise from a decrease in the rate of bone synthesis or an increase in the rate of bone destruction or both. The most common form of osteopenia is primary osteoporosis, also referred to as postmenopausal and senile osteoporosis. This form of osteoporosis is a consequence of the universal loss of bone with age and is usually a result of increase in bone resorption with a normal rate of bone formation. About 25 to 30 percent of all white females in the United States develop symptomatic osteoporosis. A

direct relationship exists between osteoporosis and the incidence of hip, femoral, neck and inter-trochanteric fracture in women 45 years and older. Elderly males develop symptomatic osteoporosis between the ages of 50 and 70, but the disease primarily affects females.

The cause of postmenopausal and senile osteoporosis is unknown. Several factors have been identified which may contribute to the condition. They include alteration in hormone levels accompanying aging and inadequate calcium consumption attributed to decreased intestinal absorption of calcium and other minerals. Treatments have usually included hormone therapy or dietary supplements in an attempt to retard the process. To date, however, an effective treatment for bone loss does not exist.

The invention provides for a method of treating a bone disorder using a therapeutically effective amount of OPG. The bone disorder may be any disorder characterized by a net bone loss (osteopenia or osteolysis). In general, treatment with OPG is anticipated when it is necessary to suppress the rate of bone resorption. Thus treatment may be done to reduce the rate of bone resorption where the resorption rate is above normal or to reduce bone resorption to below normal levels in order to compensate for below normal levels of bone formation.

Conditions which are treatable with OPG include the following:

Osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities.

Paget's disease of bone (osteitis deformans)  
in adults and juveniles

Osteomyelitis, or an infectious lesion in  
bone, leading to bone loss.

5           Hypercalcemia resulting from solid tumors  
(breast, lung and kidney) and hematologic malignancies  
(multiple myeloma, lymphoma and leukemia), idiopathic  
hypercalcemia, and hypercalcemia associated with  
hyperthyroidism and renal function disorders.

10           Osteopenia following surgery, induced by  
steroid administration, and associated with disorders  
of the small and large intestine and with chronic  
hepatic and renal diseases.

15           Osteonecrosis, or bone cell death, associated  
with traumatic injury or nontraumatic necrosis  
associated with Gaucher's disease, sickle cell anemia,  
systemic lupus erythematosus and other conditions.

Bone loss due to rheumatoid arthritis.

Periodontal bone loss.

20           Osteolytic metastasis

It is understood that OPG may be used alone  
or in conjunction with other factors for the treatment  
of bone disorders. In one embodiment, osteoprotegerin  
is used in conjunction with a therapeutically effective  
25 amount of a factor which stimulates bone formation.  
Such factors include but are not limited to the bone  
morphogenic factors designated BMP-1 through BMP-12,  
transforming growth factor- $\beta$  (TGF- $\beta$ ) and TGF- $\beta$  family  
members, interleukin-1 inhibitors, TNF $\alpha$  inhibitors,  
30 parathyroid hormone and analogs thereof, parathyroid  
related protein and analogs thereof, E series  
prostaglandins, bisphosphonates (such as alendronate  
and others), and bone-enhancing minerals such as  
fluoride and calcium.

35

The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

5

#### EXAMPLE 1

##### Identification and isolation of the rat OPG cDNA

10

Materials and methods for cDNA cloning and analysis are described in Maniatis et al, ibid. Polymerase chain reactions (PCR) were performed using a Perkin-Elmer 9600 thermocycler using PCR reaction  
15 mixture (Boehringer-Mannheim) and primer concentrations specified by the manufacturer. In general, 25-50  $\mu$ l reactions were denatured at 94°C, followed by 20-40 cycles of 94°C for 5 seconds, 50-60°C for 5 seconds, and 72°C for 3-5 minutes. Reactions were then  
20 for 72 °C for 3-5 minutes. Reactions were then analyzed by gel electrophoresis as described in Maniatis et al., ibid.

A cDNA library was constructed using mRNA isolated from embryonic d20 intestine for EST analysis  
25 (Adams et al. Science 252, 1651-1656 (1991)). Rat embryos were dissected, and the entire developing small and large intestine removed and washed in PBS. Total cell RNA was purified by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi  
30 Anal. Biochem. 162, 156-159, (1987)). The poly (A+) mRNA fraction was obtained from the total RNA preparation by adsorption to, and elution from, Dynabeads Oligo (dT)25 (Dynal Corp) using the manufacturer's recommended procedures. A random primed  
35 cDNA library was prepared using the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md). The random cDNA

primer containing an internal Not I restriction site was used to initiate first strand synthesis and had the following sequence:

5'-AAAGGAAGGAAAAAAGCGGCCGCTACANNNNNNNNT-3' (SEQ ID NO:1)

#### Not I

For the first strand synthesis three separate reactions were assembled that contained 2.5 µg of poly(A) RNA and 120 ng, 360 ng or 1,080 ng of random primer. After second strand synthesis, the reaction products were separately extracted with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1 ratio), and then ethanol precipitated. The double strand (ds) cDNA products of the three reactions were combined and ligated to the following ds oligonucleotide adapter:

5'-TCGACCCACGCGTCCG-3' (SEQ ID NO:2)

3'-GGGTGCGCAGGCp-5' (SEQ ID NO:3)

After ligation the cDNA was digested to completion with Not I, extracted with phenol:chloroform:isoamyl (25:24:1) alcohol and ethanol precipitated. The resuspended cDNA was then size fractionated by gel filtration using premade columns provided with the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md) as recommended by the manufacturer. The two fractions containing the largest cDNA products were pooled, ethanol precipitated and then directionally ligated into Not I and Sal I digested pMOB vector DNA (Strathmann et al, 1991). The ligated cDNA was introduced into competent ElectromAX DH10B E. coli (Gibco BRL, Gaithersburg, MD) by electroporation. For automated sequence analysis approximately 10,000 transformants were plated on 20cm x 20cm agar plates containing ampicillin supplemented LB nutrient media. The colonies that arose were picked

and arrayed onto 96 well microtiter plates containing 200 µl of L-broth, 7.5% glycerol, and 50 µg/ml ampicillin. The cultures were grown overnight at 37°C, a duplicate set of microtiter plates were made using a sterile 96 pin replicating tool, then both sets were stored at -80°C for further analysis. For full-length cDNA cloning approximately one million transformants were plated on 96 bacterial ampicillin plates containing about 10,000 clones each. The plasmid DNA from each pool was separately isolated using the Qiagen Plasmid Maxi Kit (Qiagen Corp., Germany) and arrayed into 96 microtiter plates for PCR analyses.

To sequence random fetal rat intestine cDNA clones, glycerol stocks were thawed, and small aliquots diluted 1:25 in distilled. Approximately 3.0 µl of diluted bacterial cultures were added to PCR reaction mixture (Boehringer-Mannheim) containing the following oligonucleotides:

5'-TGTAACGACGGCCAGT-3' (SEQ ID NO:4)  
5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO:5)

The reactions were incubated in a thermocycler (Perkin-Elmer 9600) with the following cycle conditions: 94°C for 2 minutes; 30 cycles of 94°C for 5 seconds, 50°C for 5 seconds, and 72°C for 3 minutes.; 72°C for 4 minutes. After incubation in the thermocycler, the reactions were diluted with 2.0 mL of water. The amplified DNA fragments were further purified using Centricon columns (Princeton Separations) using the manufacturer's recommended procedures. The PCR reaction products were sequenced on an Applied Biosystems 373A automated DNA sequencer using T3 primer (oligonucleotide 353-23; 5'-CAATTAACCCTCACTAAAGG-3') (SEQ ID NO:6) Taq dye-



terminator reactions (Applied Biosystems) following the manufacturer's recommended procedures.

The resulting 5' nucleotide sequence obtained from randomly picked cDNA clones translated and then compared to the existing database of known protein sequences using a modified version of the FASTA program (Pearson et al. Meth. Enzymol. 183, (1990)). Translated sequences were also analysed for the presence of a specific cysteine-rich protein motif found in all known members of the tumor necrosis factor receptor (TNFR) superfamily (Smith et al. Cell 76, 959-962 (1994)), using the sequence profile method of Gribskov et al. (Proc. Natl. Acad. Sci. USA 83, 4355-4359 (1987)), as modified by Luethy et al. (Protein Science 3, 139-146 (1994)).

Using the FASTA and Profile search data, an EST, FRI-1 (Fetal Rat Intestine-1), was identified as a possible new member of the TNFR superfamily. FRI-1 contained an approximately 600 bp insert with a LORF of about 150 amino acids. The closest match in the database was the human type II TNFR (TNFR-2). The region compared showed an ~43% homology between TNFR-2 and FRI-1 over this 150 aa LORF. Profile analysis using the first and second cysteine-rich repeats of the TNFR superfamily yielded a Z score of ~8, indicating that the FRI-1 gene possibly encodes a new family member.

To deduce the structure of the FRI-1 product, the fetal rat intestine cDNA library was screened for full length clones. The following oligonucleotides were derived from the original FRI-1 sequence:

5'-GCATTATGACCCAGAAACCGGAC-3' (SEQ ID NO:7)

5'-AGGTAGCGCCCTTCCTCACATTC-3' (SEQ ID NO:8)

These primers were used in PCR reactions to screen 96 pools of plasmid DNA, each pool containing plasmid DNA from 10,000 independent cDNA clones. Approximately 1 ug of plasmid pool DNA was amplified in a PCR reaction mixture (Boehringer-Mannheim) using a Perkin-Elmer 96 well thermal cycler with the following cycle conditions: 2 min at 94°C, 1 cycle; 15 sec at 94°C, then 45 sec at 65°C, 30 cycles; 7 min at 65°C, 1 cycle. PCR reaction products were analysed by gel electrophoresis. 13 out of 96 plasmid DNA pools gave rise to amplified DNA products with the expected relative molecular mass.

DNA from one positive pool was used to transform competent ElectromAX DH10B *E. coli* (Gibco BRL, Gaithersburg, MD) as described above. Approximately 40,000 transformants were plated onto sterile nitrocellulose filters (BA-85, Schleicher and Schuell), and then screened by colony hybridization using a <sup>32</sup>P-dCTP labelled version of the PCR product obtained above. Filters were prehybridized in 5X SSC, 50% deionized formamide, 5X Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured salmon sperm DNA for 2-4 hours at 42°C. Filters were then hybridized in 5X SSC, 50% deionized formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, and ~5 ng/ml of labelled probe for ~18 hours at 42°C. The filters were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at 55°C, and finally in 0.5X SSC for 10-15 min at 55°C. Hybridizing clones were detected following autoradiography, and then replated onto nitrocellulose filters for secondary screening. Upon secondary screening, a plasmid clone (pB1.1) was isolated, then amplified in L-broth media containing 100 ug/ml ampicillin and the plasmid DNA obtained. Both strands of the 2.4 kb pB1.1 insert were sequenced.

The pB1.1 insert sequence was used for a FASTA search of the public database to detect any existing sequence matches and/or similarities. No matches to any known genes or EST's were found, although there was an approximate 45% similarity to the human and mouse TNFR-2 genes. A methionine start codon is found at bp 124 of the nucleotide sequence, followed by a LORF encoding 401 aa residues that terminates at bp 1327. The 401 aa residue product is predicted to have a hydrophobic signal peptide of approximately 31 residues at its N-terminus, and 4 potential sites of N-linked glycosylation. No hydrophobic transmembrane spanning sequence was identified using the PepPlot program (Wisconsin GCG package, version 8.1). The deduced 401 aa sequence was then used to search the protein database. Again, there were no existing matches, although there appeared to be a strong similarity to many members of the TNFR superfamily, most notably the human and mouse TNFR-2. A sequence alignment of this novel protein with known members of the TNFR-superfamily was prepared using the Pileup program, and then modified by PrettyPlot (Wisconsin GCG package, version 8.1). This alignment shows a clear homology between the full length FRI-1 gene product and all other TNFR family members. The homologus region maps to the extracellular domain of TNFR family members, and corresponds to the three or four cysteine-rich repeats found in the ligand binding domain of these proteins. This suggested that the FRI-1 gene encoded a novel TNFR family member. Since no transmembrane spanning region was detected we predicted that this may be a secreted receptor, similar to TNFR-1 derived soluble receptors (Kohn et al. Proc. Natl. Acad. Sci. USA 87, 8331-8335 (1990)). Due to the apparent biological activity of the FRI-1 gene (*vide infra*), the product was named Osteoprotegerin (OPG).

## EXAMPLE 2

### OPG mRNA Expression Patterns in Tissues

5

Multiple human tissue northern blots (Clonetech) were probed with a  $^{32}\text{P}$ -dCTP labelled FRI-1 PCR product to detect the size of the human transcript and to determine patterns of expression. Northern  
10 blots were prehybridized in 5X SSPE, 50% formamide, 5X Denhardt's solution, 0.5% SDS, and 100  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA for 2-4 hr at 42°C. The blots were then hybridized in 5X SSPE, 50% formamide, 2X Denhardt's solution, 0.1% SDS, 100  $\mu\text{g}/\text{ml}$  denatured  
15 salmon sperm DNA, and 5 ng/ml labelled probe for 18-24 hr at 42°C. The blots were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at 50°C, then in 0.5X SSC for 10-15 min.

Using a probe derived from the rat gene, a  
20 predominant mRNA species with a relative molecular mass of about 2.4 kb is detected in several tissues, including kidney, liver, placenta, and heart. Highest levels are detected in the kidney. A large mRNA species of Mr 4.5 and 7.5 kb was detected in skeletal  
25 muscle and pancreas. In human fetal tissue, kidney was found to express relatively high levels of the 2.4 kb mRNA. Using a human probe (vide infra), only the 2.4 kb transcript is detected in these same tissues. In addition, relatively high levels of the 2.4 kb  
30 transcript was detected in the lymph node, thymus, spleen and appendix. The size of the transcript detected by both the rat and human Osteosprotegerin gene is almost identical to the length of the rat pB1.1 FRI-1 insert, suggesting it was a full length cDNA  
35 clone.

EXAMPLE 3

Systemic delivery of OPG in transgenic mice

The rat OPG clone pB1.1 was used as template  
5 to PCR amplify the coding region for subcloning into an  
ApoE-liver specific expression vector (Simonet et al.  
J. Clin. Invest. 94, 1310-1319 (1994), and PCT  
Application No. US94/11675 and co-owned U.S. Serial No.  
08/221,767. The following 5' and 3' oligonucleotide  
10 primers were used for PCR amplification, respectively:

5'-GACTAGTCCCACAATGAACAAGTGGCTGTG-3' (SEQ ID NO:9)  
5'-ATAAGAATGCGGCCGCTAAACTATGAAACAGCCCAGTGACCATTC-3'  
(SEQ ID NO:10)

15 The PCR reaction mixture (Boehringer-  
Mannheim) was treated as follows: 94°C for 1 minute, 1  
cycle; 94°C for 20 sec, 62°C for 30 sec, and 74 C for 1  
minute, 25 cycles. Following amplification, the  
20 samples were purified over Qiagen PCR columns and  
digested overnight with SpeI and NotI restriction  
enzymes. The digested products were extracted and  
precipitated and subcloned into the ApoE promoter  
expression vector. Prior to microinjecting the  
25 resulting clone, HE-OPG, it was sequenced to ensure it  
was mutation-free.

The HE-OPG plasmid was purified through two  
rounds of CsCl density gradient centrifugation. The  
purified plasmid DNA was digested with XhoI and Ase I,  
30 and the 3.6 kb transgene insert was purified by gel  
electrophoresis. The purified fragment was diluted to  
a stock injection solution of 1 µg/ml in 5 mM Tris, pH  
7.4, 0.2 mM EDTA. Single-cell embryos from BDF1 x  
BDF1-bred mice were injected essentially as described  
35 (Brinster et al., Proc. Natl. Acad. Sci. USA 82, 4338  
(1985)), except that injection needles were beveled and

siliconized before use. Embryos were cultured overnight in a CO<sub>2</sub> incubator and 15 to 20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

5           Following term pregnancy, 49 offspring were obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. The target region for amplification was a 369 bp region of the  
10 human Apo E intron which was included in the expression vector. The oligos used for PCR amplification were:

5'- GCC TCT AGA AAG AGC TGG GAC-3' (SEQ ID NO:11)

5'- CGC CGT GTT CCA TTT ATG AGC-3' (SEQ ID NO:12)

15           The conditions for PCR were: 94°C for 2 minute, 1 cycle; 94°C for 1 min, 63°C for 20 sec, and 72°C for 30 sec, 30 cycles. Of the 49 original offspring, 9 were identified as PCR positive transgenic  
20 founders.

          At 8-10 weeks of age, five transgenic founders (2, 11, 16, 17, and 28) and five controls ( 1, 12, 15, 18, and 30) were sacrificed for necropsy and pathological analysis. Liver was isolated from the  
25 remaining 4 founders by partial hepatectomy. For partial hepatectomy, the mice were anesthetized and a lobe of liver was surgically removed. Total cellular RNA was isolated from livers of all transgenic founders, and 5 negative control littermates as  
30 described (McDonald et al. Meth. Enzymol. 152, 219 (1987)). Northern blot analysis was performed on these samples to assess the level of transgene expression. Approximately 10ug of total RNA from each animal liver was resolved by electrophoresis denaturing gels (Ogden  
35 et al. Meth. Enzymol 152, 61 (1987)), then transferred to HYBOND-N nylon membrane (Amersham), and probed with

32P dCTP-labelled pB1.1 insert DNA. Hybridization was performed overnight at 42°C in 50% Formamide, 5 x SSPE, 0.5% SDS, 5 x Denhardt's solution, 100 µg/ml denatured salmon sperm DNA and 2-4 x 10<sup>6</sup> cpm of labeled probe/ml of hybridization buffer. Following hybridization, blots were washed twice in 2 x SSC, 0.1% SDS at room temperature for 5 min each, and then twice in 0.1 x SSC, 0.1% SDS at 55°C for 5-10 min each. Expression of the transgene in founder and control littermates was determined following autoradiography.

The northern blot data indicate that 7 of the transgenic founders express detectable levels of the transgene mRNA (animal #'s 2,11,16,17,22,33,and 45). The negative control mice and one of the founders (#28) expressed no transgene-related mRNA. Since OPG is predicted to be a secreted protein, overexpression of transgene mRNA should be a proxy for the level of systemically delivered gene product. Of the PCR and northern blot positive mice, animal 2, 17 and 22 expressed the highest levels of transgene mRNA, and may show more extensive biological effects on host cells and tissues.

25

#### EXAMPLE 4

##### Biological activity of OPG

Five of the transgenic mice (animals 2,11,16,17 and 28) and 5 control littermates (animals 1,12,15,18, and 30) were sacrificed for necropsy and pathological analysis using the following procedures: Prior to euthanasia, all animals had their identification numbers verified, then were weighed, anesthetized and blood drawn. The blood was saved as both serum and whole blood for a complete serum chemistry and hematology panel. Radiography was

performed just after terminal anesthesia by lethal CO<sub>2</sub> inhalation, and prior to the gross dissection.

Following this, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The

5 tissues collected included the liver, spleen, pancreas, stomach, duodenum, ileum, colon, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, esophagus, thyroid, jejunum, cecum, rectum, adrenals, urinary bladder, and skeletal  
10 muscle. Prior to fixation the whole organ weights were determined for the liver, stomach, kidney, adrenals, spleen, and thymus. After fixation the tissues were processed into paraffin blocks, and 3  $\mu$ m sections were obtained. Bone tissue was decalcified using a formic  
15 acid solution, and all sections were stained with hematoxylin and eosin. In addition, staining with Gomori's reticulin and Masson's trichrome were performed on certain tissues. Enzyme histochemistry was performed to determine the expression of tartrate  
20 resistant acid phosphatase (TRAP), an enzyme highly expressed by osteoclasts, multinucleated bone-resorbing cells of monocyte-macrophage lineage.

Immunohistochemistry for BrdU and F480 monocyte-macrophage surface antigen was also performed to detect  
25 replicating cells and cells of the monocyte-macrophage lineage, respectively. To detect F480 surface antigen expression, formalin fixed, paraffin embedded 4  $\mu$ m sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen  
30 peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-mouse F480 (Harlan, Indianapolis, IN). This antibody was detected by biotinylated rabbit anti-rat immunoglobulins, peroxidase conjugated streptavidin  
35 (BioGenex San Ramon, CA) with DAB as chromagen (BioTek,



Santa Barbara, CA). Sections were counterstained with hematoxylin.

Upon gross dissection and observation of visceral tissues, no abnormalities were found in the transgene expressors or control littermates. Analysis of organ weight indicate that spleen size increased by approximately 38% in the transgenic mice relative to controls. There was a slight enlargement of platelet size and increased circulating unstained cells in the transgene expressors. There was a marginal decrease in platelet levels in the transgene expressors. In addition, the serum uric acid, urea nitrogen, and alkaline phosphatase levels all trended lower in the transgene expressors. The expressors were found to have increased radiodensity of the skeleton, including long bones (femurs), vertebrae, and flat bones (pelvis). The relative size of femurs in the expressors were not different from the the control mice.

Histological analysis of stained sections of bone from the OPG expressors show severe osteopetrosis with the presence of cartilage remnants from the primary spongiosa seen within bone trabeculae in the diaphysis of the femur. A clearly defined cortex was not identifiable in the sections of femur. In normal animals, the central diaphysis is filled with bone marrow. Sections of vertebra also show osteopetrotic changes implying that the OPG-induced skeletal changes were systemic. The residual bone marrow showed predominantly myeloid elements. Megakaryocytes were present. Reticulin stains showed no evidence for reticulin deposition. Immunohistochemistry for F480, a cell surface antigen expressed by cells of monocyte-macrophage derivation in the mouse, showed the presence of F480 positive cells in the marrow spaces. Focally,

flattened F480 positive cells could be seen directly adjacent to trabecular bone surfaces.

5 The mesenchymal cells lining the bony  
trabeculae were flattened and appeared inactive. Based  
on H&E and TRAP stains, osteoclasts were rarely found  
10 on the trabecular bone surfaces in the OPG expressors.  
In contrast, osteoclasts and/or chondroclasts were seen  
in the region of the growth plate resorbing cartilage,  
but their numbers may be reduced compared to controls.  
Also, osteoclasts were present on the cortical surface  
of the metaphysis where modelling activity is usually  
robust. The predominant difference between the  
expressors and controls was the profound decrease in  
15 trabecular osteoclasts, both in the vertebrae and  
femurs. The extent of bone accumulation was directly  
correlated with the level of OPG transgene mRNA  
detected by northern blotting of total liver RNA.

The spleens from the OPG expressors had an  
increased amount of red pulp with the expansion due to  
20 increased hematopoiesis. All hematopoietic lineages are  
represented. F480 positive cells were present in both  
control and OPG expressors in the red pulp. Two of the  
expressors (2 and 17) had foci of extramedullary  
hematopoiesis within the liver and this is likely due  
25 to the osteopetrotic marrow.

There were no observable abnormalities in  
the thymus, lymph nodes, gastrointestinal tract,  
pancreato-hepatobiliary tract, respiratory tract,  
reproductive system, genito-urinary system, skin,  
30 nervous system, heart and aorta, breast, skeletal  
muscle and fat.

EXAMPLE 5

Isolation of mouse and human OPG cDNA

5 A cDNA clone corresponding to the 5' end of the mouse OPG mRNA was isolated from a mouse kidney cDNA library (Clontech) by PCR amplification. The oligonucleotides were derived from the rat OPG cDNA sequence and are shown below:

- 10 5'-ATCAAAGGCAGGGCATACTTCCTG-3' (SEQ ID NO:13)  
5'-GTTGCACTCCTGTTTCACGGTCTG-3' (SEQ ID NO:14)
- 5'-CAAGACACCTTGAAGGGCCTGATG-3' (SEQ ID NO:15)  
5'-TAACTTTTACAGAAGAGCATCAGC-3' (SEQ ID NO:16)
- 15 5'-AGCGCGGCCCGCATGAACAAGTGGCTGTGCTGCG-3' (SEQ ID NO:17)  
5'-AGCTCTAGAGAAACAGCCCAGTGACCATTCC-3' (SEQ ID NO:18)

20 The partial and full-length cDNA products obtained in this process were sequenced. The full-length product was digested with Not I and Xba I, then directionally cloned into the plasmid vector pRcCMV (Invitrogen). The resulting plasmid was named pRcCMV-Mu-OPG. The nucleotide sequence of the cloned product

25 was compared to the rat OPG cDNA sequence. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA sequences are approximately 88% identical. The mouse cDNA sequence contained a 401 aa LORF, which was compared to the rat OPG protein sequence and found to

30 be ~94% identical without gaps. This indicates that the mouse cDNA sequence isolated encodes the murine OPG protein, and that the sequence and structure has been highly conserved throughout evolution. The mouse OPG protein sequence contains an identical putative signal

35 peptide at its N-terminus, and all 4 potential sites of N-linked glycosylation are conserved.

A partial human OPG cDNA was cloned from a human kidney cDNA library using the following rat-specific oligonucleotides:

5 5'-GTG AAG CTG TGC AAG AAC CTG ATG-3'

(SEQ ID NO:19)

5'-ATC AAA GGC AGG GCA TAC TTC CTG-3'

(SEQ ID NO:20)

10 This PCR product was sequenced and used to design primers for amplifying the 3' end of the human cDNA using a human OPG genomic clone in lambda as template:

15 5'-TCCGTAAGAAACAGCCCAGTGACC-3' (SEQ ID NO:29)

5'-CAGATCCTGAAGCTGCTCAGTTTG-3' (SEQ ID NO:21)

20 The amplified PCR product was sequenced, and together with the 5' end sequence, was used to design 5' and 3' human-specific primers useful for amplifying the entire human OPG cDNA coding sequences:

5'-AGCGCGGCCGCGGGGACCACAATGAACAAGTTG-3' (SEQ ID NO:22)

5'-AGCTCTAGAATTGTGAGGAAACAGCTCAATGGC-3' (SEQ ID NO:23)

25

The full-length human PCR product was sequenced, then directionally cloned into the plasmid vector pRcCMV (Invitrogen) using Not I and Xba I. The resulting plasmid was named pRcCMV-human OPG. The nucleotide sequence of the cloned product was compared to the rat and mouse OPG cDNA sequences. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA sequences are approximately 78-88% identical to the human OPG cDNA. The human OPG cDNA sequence also contained a 401 aa LORF, and it was compared to the rat and mouse protein sequences. The predicted human OPG

protein is approximately 85% identical, and ~90% identical to the rat and mouse proteins, respectively. Sequence alignment of rat, mouse and human proteins show that they have been highly conserved during evolution. The human protein is predicted to have a N-terminal signal peptide, and 5 potential sites of N-linked glycosylation, 4 of which are conserved between the rat and mouse OPG proteins.

The DNA and predicted amino acid sequence of mouse OPG is shown in Figure 9A and 9B (SEQ ID NO:122). The DNA and predicted amino acid sequence of human OPG is shown in Figure 9C and 9D (SEQ ID NO:124). A comparison of the rat, mouse and human OPG amino acid sequences is shown in Figure 9E and 9F.

Isolation of additional human OPG cDNA clones revealed the presence of a G to C base change at position 103 of the DNA sequence shown in Figure 9C. This nucleotide change results in substitution of an asparagine for a lysine at position 3 of the amino acid sequence shown in Figure 9C. The remainder of the sequence in clones having this change was identical to that in Figure 9C and 9D.

25

#### EXAMPLE 6

##### OPG three-dimensional structure modelling

The amino-terminal portion of OPG has homology to the extracellular portion of all known members of the TNFR superfamily (Figure 1C). The most notable motif in this region of TNFR-related genes is an ~40 amino acid, cysteine-rich repeat sequence which folds into distinct structures (Banner et al. Cell 73, 431-445 (1993)). This motif is usually displayed in four (range 3-6) tandem repeats (see Figure 1C), and is known to be involved in ligand binding (Beutler and van

Huffel Science 264, 667-663 (1994)). Each repeat usually contains six interspaced cysteine residues, which are involved in forming three intradomain disulfide bonds, termed SS1, SS2, and SS3 (Banner et al., ibid). In some receptors, such as TNFR2, CD30 and CD40, some of the repeat domains contain only two intrachain disulfide bonds (SS1 and SS3).

The human OPG protein sequence was aligned to a TNFR1 extracellular domain profile using methods described by Luethy, et al., ibid, and the results were graphically displayed using the PrettyPlot program from the Wisconsin Package, version 8.1 (Genetics Computer Group, Madison, WI) (Figure 10). The alignment indicates a clear conservation of cysteine residues involved in formation of domains 1-4. This alignment was then used to construct a three-dimensional (3-D) model of the human OPG N-terminal domain using the known 3-D structure of the extracellular domain of p55 TNFR1 (Banner et al., ibid) as the template. To do this the atomic coordinates of the peptide backbone and side chains of identical residues were copied from the crystal structure coordinates of TNFR1. Following this, the remaining coordinates for the insertions and different side chains were generated using the LOOK program (Molecular Applications Group, Palo Alto, CA). The 3-D model was then refined by minimizing its conformational energy using LOOK.

By analogy with other TNFR family members, it is assumed that OPG binds to a ligand. For the purpose of modelling the interaction of OPG with its ligand, the crystal structure of TNF- $\beta$  was used to simulate a 3-D representation of an "OPG ligand". This data was graphically displayed (see Figure 11) using Molscript (Kraulis, J. Appl. Cryst. 24, 946-950, 1991). A model for the OPG/ligand complex with 3 TNF $\beta$  and 3 OPG molecules was constructed where the relative positions

of OPG are identical to TNFR1 in the crystal structure. This model was then used to find the residues of OPG that could interact with its ligand using the following approach: The solvent accessible area of all residues  
5 in the complex and one single OPG model were calculated. The residues that have different accessibility in the complex than in the monomer are likely to interact with the ligand.

The human and mouse OPG amino acid sequences  
10 were realigned using this information to highlight sequences comprising each of the cysteine rich domains 1-4 (Figure 12A and 12B). Each domain has individual structural characteristics which can be predicted:

15       Domain 1

Contains 4 cysteines involved in SS2 (C41 to C54) and SS3 (C44 to C62) disulfide bonds. Although no SS1 bond is evident based on disulfide bridges, the conserved tyrosine at position 28 is homologous to Y20  
20 in TNFR1, which is known to be involved in interacting with H66 to aid in domain formation. OPG has a homologous histidine at position 75, suggesting OPG Y28 and H75 stack together in the native protein, as do the homologous residues in TNFR1. Therefore, both of these  
25 residues may indeed be important for biological activity, and N-terminal OPG truncations up to and beyond Y28 may have altered activity. In addition, residues E34 and K43 are predicted to interact with a bound ligand based on our 3-dimensional model.

30

Domain 2

Contains six cysteines and is predicted to contain SS1 (C65 to C80), SS2 (C83 to C98) and SS3 (C87 to C105) disulfide bonds. This region of OPG also  
35 contains an region stretching from P66-Q91 which aligns to the portion of TNFR1 domain 2 which forms close

contacts with  $\text{TNF}\beta$  (see above), and may interact with an OPG ligand. In particular residues P66, H68, Y69, Y70, T71, D72, S73, H75, T76, S77, D78, E79, L81, Y82, P85, V86, K88, E89, L90, and Q91 are predicted to  
5 interact with a bound ligand based on our structural data.

#### Domain 3

Contains 4 cysteines involved in SS1 (C107 to  
10 C 118) and SS3 (C124 to C142) disulfide bonds, but not an SS2 bond. Based on our structural data, residues E115, L118 and K119 are predicted in to interact with an OPG ligand.

#### Domain 4

Contains 4 cysteines involved in SS1 (C145 to  
15 C160) and SS3 (C166 to C185) disulfide bonds, but not an SS2 bond, similar to domain 3. Our structural data predict that E153 and S155 interact with an OPG ligand.  
20

Thus, the predicted structural model for OPG identifies a number of highly conserved residues which are likely to be important for its biological activity.

25

#### EXAMPLE 7

##### Production of recombinant secreted OPG protein in mammalian cells

To determine if OPG is actually a secreted  
30 protein, mouse OPG cDNA was fused to the human IgG1 Fc domain as a tag (Capon et al. Nature 337, 525-531 (1989)), and expressed in human 293 fibroblasts. Fc fusions were carried out using the vector pFc-A3. pFc-  
35 A3 contains the region encoding the Fc portion of human



immunoglobulin IgG- $\gamma$ 1 heavy chain (Ellison et al. ibid)  
from the first amino acid of the hinge domain (Glu-99)  
to the carboxyl terminus and is flanked by a 5'-NotI  
fusion site and 3'-SalI and XbaI sites. The plasmid  
5 was constructed by PCR amplification of the human  
spleen cDNA library (Clontech). PCR reactions were in a  
final volume of 100  $\mu$ l and employed 2 units of Vent DNA  
polymerase (New England Biolabs) in 20 mM Tris-HCl (pH  
8.8), 10 mM KCl, 10  $\mu$ M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1%  
10 Triton X-100 with 400  $\mu$ M each dNTP and 1 ng of the cDNA  
library to be amplified together with 1  $\mu$ M of each  
primer. Reactions were initiated by denaturation at  
95°C for 2 min, followed by 30 cycles of 95°C for 30 s,  
55°C for 30 s, and 73°C for 2 min. The 5' primer  
15 5' ATAGCGGCCGCTGAGCCCAAATCTTGTGACAAAACACTCAC 3' (SEQ  
ID NO:24)  
incorporated a NotI site immediately 5' to the first  
residue (Glu-99) of the hinge domain of IgG- $\gamma$ 1. The 3'  
primer  
20 5'-TCTAGAGTCGACTTATCATTTACCCGGAGACAGGGAGAGGCTCTT-  
3' (SEQ ID NO:25)  
incorporated SalI and XbaI sites. The 717-bp PCR  
product was digested with NotI and SalI, isolated by  
electrophoresis through 1% agarose (FMC Corp.), purified  
25 by the Geneclean procedure (BIO 101, Inc.) and cloned  
into NotI, SalI-digested pBluescript II KS vector  
(Stratagene). The insert in the resulting plasmid, pFc-  
A3, was sequenced to confirm the fidelity of the PCR  
reaction.  
30 The cloned mouse cDNA in plasmid pRcCMV-MuOPG  
was amplified using the following two sets of primer  
pairs:

Pair 1

5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3' (SEQ ID NO:26)

5'-CCTCTGCGGCCGCTAAGCAGCTTATTTTCACGGATTGAACCTG-3' (SEQ ID NO:27)

5

Pair 2

5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3' (SEQ ID NO:28)

10 5'-CCTCTGCGGCCGCTGTTGCATTTCTTTCTG-3' (SEQ ID NO:30)

The first pair amplifies the entire OPG LORF, and creates a NotI restriction site which is compatible with the in-frame Not I site in Fc fusion vector pFcA3. pFcA3 was prepared by engineering a NotI restriction site 5' to aspartic acid residue 216 of the human IgG1 Fc cDNA. This construct introduces a linker which encodes two irrelevant amino acids which span the junction between the OPG protein and the IgG Fc region. This product, when linked to the Fc portion, would encode all 401 OPG residues directly followed by all 227 amino acid residues of the human IgG1 Fc region (Fl.Fc). The second primer pair amplifies the DNA sequences encoding the first 180 amino acid residues of OPG, which encompasses its putative ligand binding domain. As above, the 3' primer creates an artificial Not I restriction site which fuses the C-terminal truncated OPG LORF at position threonine 180 directly to the IgG1 Fc domain (CT.fc).

The amino acid sequence junction linking OPG residue 401 and aspartic acid residue 221 of the human Fc region can be modified as follows: The DNA encoding residues 216-220 of the human Fc region can be deleted as described below, or the cysteine residue corresponding to C220 of the human Fc region can be mutated to either serine or alanine. OPF-Fc fusion

protein encoded by these modified vectors can be transfected into human 293 cells, or CHO cells, and recombinant OPG-Fc fusion protein purified as described below.

5 Both products were directionally cloned into the plasmid vector pCEP4 (Invitrogen). pCEP4 contains the Epstein-Barr virus origin of replication, and is capable of episomal replication in 293-EBNA-1 cells. The parent pCEP4, and pCEP4-F1.Fc and pCEP4-CT.Fc  
10 vectors were lipofected into 293-EBNA-1 cells using the manufacturer's recommended methods. The transfected cells were then selected in 100 µg/ml hygromycin to select for vector expression, and the resulting drug-resistant mass cultures were grown to confluence. The  
15 cells were then cultured in serum-free media for 72 hr, and the conditioned media removed and analysed by SDS-PAGE. A silver staining of the polyacrylamide gel detects the major conditioned media proteins produced by the drug resistant 293 cultures. In the pCEP4-F1.Fc  
20 and the pCEP4-CT.Fc conditioned media, unique bands of the predicted sizes were abundantly secreted (see Figures 13B and 13C). The full-length Fc fusion protein accumulated to a high concentration, indicating that it may be stable. Both Fc fusion proteins were  
25 detected by anti-human IgG1 Fc antibodies (Pierce) on western blots, indicating that they are recombinant OPG products.

The full length OPG-Fc fusion protein was purified by Protein-A column chromatography (Pierce)  
30 using the manufacturers recommended procedures. The protein was then subjected to N-terminal sequence analysis by automated Edman degradation as essentially described by Matsudaira et al. (J. Biol. Chem. 262, 10-35 (1987)). The following amino acid sequence was read  
35 after 19 cycles:

NH2-E T L P P K Y L H Y D P E T G H Q L L-CO2H  
(SEQ ID NO:31)

5                   This sequence was identical to the predicted  
mouse OPG amino acid sequence beginning at amino acid  
residue 22, suggesting that the natural mammalian  
leader cleavage site is between amino acid residues  
Q21-E22, not between Y31-D32 as originally predicted.  
10   The expression experiments performed in 293-EBNA cells  
with pCEP4-Fl.Fc and pCEP4-CT.Fc demonstrate that OPG  
is a secreted protein, and may act systemically to bind  
its ligand.

                  Procedures similar to those used to construct  
15   and express the muOPG[22-180]-Fc and muOPG[22-401]-Fc  
fusions were employed for additional mouse and human  
OPG-Fc fusion proteins.

                  Murine OPG cDNA encoding amino acids 1-185  
fused to the Fc region of human IgG1 [muOPG Ct(185).Fc]  
20   was constructed as follows. Murine OPG cDNA from  
plasmid pRcCMV Mu Osteoprotegerin (described in Example  
5) was amplified using the following primer pair in a  
polymerase chain reaction as described above:

25   1333-82:

          5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:32)

1333-80:

          5'-CCT CTG CGG CCG CAC ACA CGT TGT CAT GTG TTG C-  
3' (SEQ ID NO:33)

30

                  This primer pair amplifies the murine OPG  
cDNA region encoding amino acid residues 63-185  
(corresponding to bp 278-645) of the OPG reading frame  
as shown in Figure 9A. The 3' primer contains a Not I  
35   restriction site which is compatible with the in-frame  
Not I site of the Fc fusion vector pFcA3. The product

also spans a unique EcoRI restriction site located at  
bp 436. The amplified PCR product was purified,  
cleaved with NotI and EcoRI, and the resulting EcoRI-  
NotI restriction fragment was purified. The vector  
5 pCEP4 having the murine 1-401 OPG-Fc fusion insert was  
cleaved with EcoRI and NotI, purified, and ligated to  
the PCR product generated above. The resulting pCEP4-  
based expression vector encodes OPG residues 1-185  
directly followed by all 227 amino acid residues of the  
10 human IgG1 Fc region. The murine OPG 1-185.Fc fusion  
vector was transfected into 293 cells, drug selected,  
and conditioned media was produced as described above.  
The resulting secreted murine OPG 1-185.Fc fusion  
product was purified by Protein-A column chromatography  
15 (Pierce) using the manufacturers recommended  
procedures.

Murine OPG DNA encoding amino acid residues  
1-194 fused to the Fc region of human IgG1 (muOPG  
20 Ct(194).Fc) was constructed as follows. Mouse OPG cDNA  
from plasmid pRcCMV Mu-Osteoprotegerin was amplified  
using the following primer pairs:

1333-82:  
25 5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:34)  
1333-81:  
5'-CCT CTG CGG CCG CCT TTT GCG TGG CTT CTC TGT T-  
3' (SEQ ID NO:35)

30 This primer pair amplifies the murine OPG  
cDNA region encoding amino acid residues 70-194  
(corresponding to bp 298-672) of the OPG reading frame.  
The 3' primer contains a Not I restriction site which  
is compatible with the in-frame Not I site of the Fc  
35 fusion vector pFcA3. The product also spans a unique  
EcoRI restriction site located at bp 436. The

amplified PCR product was cloned into the murine OPG[1-401] Fc fusion vector as described above. The resulting pCEP4-based expression vector encodes OPG residues 1-194 directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-194.Fc fusion vector was transfected into 293 cells, drug selected, and conditioned media was produced. The resulting secreted fusion product was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures.

Human OPG DNA encoding amino acids 1-401 fused to the Fc region of human IgG1 was constructed as follows. Human OPG DNA in plasmid pRcCMV-hu osteoprotegerin (described in Example 5) was amplified using the following oligonucleotide primers:

1254-90:

5'CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT G-3'

(SEQ ID NO:36)

1254-95:

5'-CCT CTG CGG CCG CTA AGC AGC TTA TTT TTA CTG AAT GG-3'  
(SEQ ID NO:37)

The resulting PCR product encodes the full-length human OPG protein and creates a Not I restriction site which is compatible with the in-frame Not I site Fc fusion vector FcA3. The PCR product was directionally cloned into the plasmid vector pCEP4 as described above. The resulting expression vector encodes human OPG residues 1-401 directly followed by 227 amino acid residues of the human IgG1 Fc region. Conditioned media from transfected and drug selected cells was produced and the huOPG F1.Fc fusion product was purified by Protein-A column chromatography

(Pierce) using the manufacturers recommended procedures.

Human OPG DNA encoding amino acid residues 1-201 fused to the Fc region of human IgG1 [huOPG Ct(201).Fc] was constructed as follows. The cloned human OPG cDNA from plasmid pRrCMV-hu osteoprotegerin was amplified by PCR using the following oligonucleotide primer pair:

10

1254-90:

5'-CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT  
G-3' (SEQ ID NO:38)

1254-92:

15

5'-CCT CTG CGG CCG CCA GGG TAA CAT CTA TTC CAC-3'  
(SEQ ID NO:39)

This primer pair amplifies the human OPG cDNA region encoding amino acid residues 1-201 of the OPG reading frame, and creates a Not I restriction site at the 3' end which is compatible with the in-frame Not I site Fc fusion vector FcA3. This product, when linked to the Fc portion, encodes OPG residues 1-201 directly followed by all 221 amino acid residues of the human IgG1 Fc region. The PCR product was directionally cloned into the plasmid vector pCEP4 as described above. Conditioned media from transfected and drug selected cells was produced, and the hu OPG Ct(201).Fc fusion products purified by Protein-A column chromatography (Pierce) using the manufacturer's recommended procedures.

The following procedures were used to construct and express unfused mouse and human OPG.

35

A plasmid for mammalian expression of full-length murine OPG (residues 1-401) was generated by PCR

amplification of the murine OPG cDNA insert from pRcCMV  
Mu-Osteoprotegerin and subcloned into the expression  
vector pDSR $\alpha$  (DeClerck et. al. J. Biol. Chem. 266,  
3893 (1991)). The following oligonucleotide primers  
5 were used:

1295-26:

5'-CCG AAG CTT CCA CCA TGA ACA AGT GGC TGT GCT  
GC-3' (SEQ ID NO:40)

10

1295-27:

5'-CCT CTG TCG ACT ATT ATA AGC AGC TTA TTT TCA CGG  
ATT G-3' (SEQ ID NO:41)

15

The murine OPG full length reading frame was  
amplified by PCR as described above. The PCR product  
was purified and digested with restriction  
endonucleases Hind III and Xba I (Boehringer Mannheim,  
Indianapolis, IN) under the manufacturers recommended  
20 conditions, then ligated to Hind III and Xba I digested  
pDSR $\alpha$ . Recombinant clones were detected by restriction  
endonuclease digestion, then sequenced to ensure no  
mutations were produced during the PCR amplification  
steps.

25

The resulting plasmid, pDSR $\alpha$ -muOPG was  
introduced into Chinese hamster ovary (CHO) cells by  
calcium mediated transfection (Wigler et al. Cell 11,  
233 (1977)). Individual colonies were selected based  
upon expression of the dihydrofolate reductase (DHFR)  
30 gene in the plasmid vector and several clones were  
isolated. Expression of the murine OPG recombinant  
protein was monitored by western blot analysis of CHO  
cell conditioned media. High expressing cells were  
selected, and OPG expression was further amplified by  
35 treatment with methotrexate as described (DeClerck et  
al., idid). Conditioned media from CHO cell lines was



produced for further purification of recombinant secreted murine OPG protein.

5 A plasmid for mammalian expression of full-length human OPG (amino acids 1-401) was generated by subcloning the cDNA insert in pRcCMV-hu Osteoprotegerin directly into vector pDSR $\alpha$  (DeClerck et al., *ibid*). The pRcCMV-OPG plasmid was digested to completion with Not I, blunt ended with Klenow, then digested to completion  
10 with Xba I. Vector DNA was digested with Hind III, blunt ended with Klenow, then digested with Xba I, then ligated to the OPG insert. Recombinant plasmids were then sequenced to confirm proper orientation of the human OPG cDNA.

15 The resulting plasmid pDSR $\alpha$ -huOPG was introduced into Chinese hamster ovary (CHO) cells as described above. Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones  
20 were isolated. Expression of the human OPG recombinant protein was monitored by western blot analysis of CHO cell conditioned media. High expressing clones were selected, and OPG expression was further amplified by treatment with methotrexate. Conditioned media from  
25 CHO cell lines expressing human OPG was produced for protein purification.

Expression vectors for murine OPG encoding residues 1-185 were constructed as follows. Murine OPG  
30 cDNA from pRcCMV-Mu OPG was amplified using the following oligonucleotide primers:

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:42).

35 1356-12:

5'-CCT CTG TCG ACT TAA CAC ACG TTG TCA TGT GTT  
GC-3' (SEQ ID NO:43)

This primer pair amplifies the murine OPG  
5 cDNA region encoding amino acids 63-185 of the OPG  
reading frame (bp 278-645) and contains an artificial  
stop codon directly after the cysteine codon (C185),  
which is followed by an artificial Sal I restriction  
endonuclease site. The predicted product contains an  
10 internal Eco RI restriction site useful for subcloning  
into a pre-existing vector. After PCR amplification,  
the resulting purified product was cleaved with Eco RI  
and Sal I restriction endonucleases, and the large  
fragment was gel purified. The purified product was  
15 then subcloned into the large restriction fragment of  
an Eco RI and Sal I digest of pBluescript-muOPG Fl.Fc  
described above. The resulting plasmid was digested  
with Hind III and Xho I and the small fragment was gel  
purified. This fragment, which contains a open reading  
20 frame encoding residues 1-185 was then subcloned into a  
Hind III and Xho I digest of the expression vector  
pCEP4. The resulting vector, pmuOPG [1-185], encodes a  
truncated OPG polypeptide which terminates at a  
cysteine residue located at position 185. Conditioned  
25 media from transfected and drug selected cells was  
produced as described above.

1333-82:

30 5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:44)

1356-13:

5'-CCT CTG TCG ACT TAC TTT TGC GTG GCT TCT CTG  
TT-3' (SEQ ID NO:45)

35

This primer pair amplifies the murine OPG cDNA region encoding amino acids 70-194 of the OPG reading frame (bp 298-672) and contains an artificial stop codon directly after the lysine codon (K194), which is followed by an artificial Sal I restriction endonuclease site. The predicted product contains an internal Eco RI restriction site useful for subcloning into a pre-existing vector. After PCR amplification, the resulting purified product was cleaved with Eco RI and Sal I restriction endonucleases, and the large fragment was gel purified. The purified product was then subcloned into the large restriction fragment of an Eco RI and Sal I digest of pBluescript-muOPG F1.Fc described above. The resulting plasmid was digested with Hind III and Xho I and the small fragment was gel purified. This fragment, which contains a open reading frame encoding residues 1-185 was then subcloned into a Hind III and Xho I digest of the expression vector pCEP4. The resulting vector, pmuOPG [1-185], encodes a truncated OPG polypeptide which terminates at a lysine at position 194. Conditioned media from transfected and drug selected cells was produced as described above.

Several mutations were generated at the 5' end of the huOPG [22-401]-Fc gene that introduce either amino acid substitutions, or deletions, of OPG between residues 22 through 32. All mutations were generated with the "QuickChange™ Site-Directed Mutagenesis Kit" (Stratagene, San Diego, CA) using the manufacturer's recommended conditions. Briefly, reaction mix containing huOPG [22-401]-Fc plasmid DNA template and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent E. coli

XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to delete residues 22-26 of the human OPG gene, resulting in the production of a huOPG [27-401]-Fc fusion protein:

1436-11:

5'-TGG ACC ACC CAG AAG TAC CTT CAT TAT GAC-3'  
(SEQ ID NO:140)

1436-12:

5'-GTC ATA ATG AAG GTA CTT CTG GGT GGT CCA-3'  
(SEQ ID NO:141)

The following primer pairs were used to delete residues 22-28 of the human OPG gene, resulting in the production of a huOPG [29-401]-Fc fusion protein:

1436-17:

5'-GGA CCA CCC AGC TTC ATT ATG ACG AAG AAA C-3'  
(SEQ ID NO:142)

1436-18:

5'-GTT TCT TCG TCA TAA TGA AGC TGG GTG GTC C-3'  
(SEQ ID NO:143)

The following primer pairs were used to delete residues 22-31 of the human OPG gene, resulting in the production of a huOPG [32-401]-Fc fusion protein:

1436-27:

5'-GTG GAC CAC CCA GGA CGA AGA AAC CTC TC-3'  
(SEQ ID NO:144)

5 1436-28:

5'-GAG AGG TTT CTT CGT CCT GGG TGG TCC AC-3'  
(SEQ ID NO:145)

The following primer pairs were used to  
10 change the codon for tyrosine residue 28 to  
phenylalanine of the human OPG gene, resulting in the  
production of a huOPG [22-401]-Fc Y28F fusion protein:

1436-29:

15 5'-CGT TTC CTC CAA AGT TCC TTC ATT ATG AC-3'  
(SEQ ID NO:146)

1436-30:

20 5'-GTC ATA ATG AAG GAA CTT TGG AGG AAA CG-3'  
(SEQ ID NO:147)

The following primer pairs were used to  
change the codon for proline residue 26 to alanine of  
the human OPG gene, resulting in the production of a  
25 huOPG [22-401]-Fc P26A fusion protein:

1429-83:

5'-GGA AAC GTT TCC TGC AAA GTA CCT TCA TTA TG-3  
(SEQ ID NO:148)

30

1429-84:

5'-CAT AAT GAA GGT ACT TTG CAG GAA ACG TTT CC-3'  
(SEQ ID NO:149)

35

Each resulting muOPG [22-401]-Fc plasmid  
containing the appropriate mutation was then

transfected into human 293 cells, the mutant OPG-Fc  
fusion protein purified from conditioned media as  
described above. The biological activity of each  
protein was assessed the in vitro osteoclast forming  
5 assay described in Example 11.

#### EXAMPLE 8

##### Expression of OPG in E. coli

10

##### A. Bacterial Expression Vectors

##### pAMG21

The expression plasmid pAMG21 can be derived  
15 from the Amgen expression vector pCFM1656 (ATCC #69576)  
which in turn be derived from the Amgen expression  
vector system described in US Patent No. 4,710,473.  
The pCFM1656 plasmid can be derived from the described  
pCFM836 plasmid (Patent No. 4,710,473) by: (a)  
20 destroying the two endogenous NdeI restriction sites  
by end filling with T4 polymerase enzyme followed by  
blunt end ligation; (b) replacing the DNA sequence  
between the unique AatII and ClaI restriction sites  
containing the synthetic P<sub>L</sub> promoter with a similar  
25 fragment obtained from pCFM636 (patent No. 4,710,473)  
containing the PL promoter

AatII  
30 5' CTAATTCCGCTCTCACCTACCAAACAATGCCCCCTGCAAAAAATAAATTCATAT-  
3' TGCAGATTAAGGCGAGAGTGGATGGTTTGTACGGGGGACGTTTTTTATTTAAGTATA-

-AAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-  
-TTTTTTGTATGTCTATTGGTAGACGCCACTATTTAATAGAGACCGCCACAACGTATT-

35

-TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)  
-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

ClaI

and then (c) substituting the small DNA sequence between the unique *ClaI* and *KpnI* restriction sites with the following oligonucleotide:

5  
5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC3'  
(SEQ ID NO:48)  
3' TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC 5'  
10 (SEQ ID NO:49)  
*ClaI* *KpnI*

The expression plasmid pAMG21 can then be derived from pCFM1656 by making a series of site directed base  
15 changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the *BglIII* site (plasmid bp # 180) immediately 5' to the plasmid replication promoter *PcopB* and proceeding toward the plasmid replication genes, the base pair changes are as  
20 follows:

	<u>pAMG21 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG21</u>
25	# 204	T/A	C/G
	# 428	A/T	G/C
	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
	# 679	G/C	T/A
	# 980	T/A	C/G
30	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
	# 1028	A/T	T/A
	# 1047	C/G	T/A
35	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
	# 2480	A/T	T/A
40	# 2499-2502	AGTG TCAC	GTCA CAGT
45	# 2642	TCCGAGC AGGCTCG	7 bp deletion
	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A

The DNA sequence between the unique AatII (position  
#4364 in pCFM1656) and SacII (position #4585 in  
5 pCFM1656) restriction sites is substituted with the  
following DNA sequence:

[AatII sticky end] 5' GCGTAACGTATGCATGGTCTCC-  
10 (position #4358 in pAMG21) 3' TGCACGCATTGCATACGTACCAGAGG-  
-CCATGCGAGAGTAGGGAAGTCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACT-  
-GGTACGCTCTCATCCCTTGACGGTCCGTAGTTTATTTTGCTTTCCGAGTCAGCTTTCTGA-  
15 -GGGCCCTTTCGTTTATCTGTTGTTTGTGCGGTGAACGCTCTCCTGAGTAGGACAAATCCGC-  
-CCCGGAAAGCAAAATAGACAACAAACAGCCACTTGCGAGAGGACTCATCTGTTTAGGCG-  
-CGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCCGGAGGGTGGCGGGCAGGACGCCCCG-  
-GCCCTCGCCTAAACTTGCAACGCTTCGTTGCCGGGCCTCCACCGCCCGTCTGCGGGCG-  
20 -CATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGT-  
-GTATTTGACGGTCCGTAGTTTAAATTCGTCTTCCGGTAGGACTGCCTACCGGAAAAACGCA-  
AatII  
25 -TTCTACAACTCTTTTGTATTTTCTAAATACATTCAAATATGGACGTCGTAATTAAC-  
-AAGATGTTTGAGAAAACAAATAAAAAGATTTATGTAAGTTTATACCTGCAGCATGAATTG-  
-TTTTAAAGTATGGGCAATCAATTGCTCCTGTAAAATTGCTTTAGAAATACTTTGGCAGC-  
-AAAATTTCAACCCGTTAGTTAACGAGGACAATTTTAACGAAATCTTTATGAAACCGTCG-  
30 -GGTTTGTGTATTGAGTTTCATTTGCGCATTTGGTTAAATGGAAAGTGACCGTGCGCTTAC-  
-CCAAACAACATAACTCAAAGTAAACGCGTAACCAATTTACCTTTCACTGGCACGCGAATG-  
-TACAGCCTAATATTTTGAATATCCCAAGAGCTTTTTCTTCGATGCCACGCTAAAC-  
35 -ATGTCGGATTATAAAAACCTTTATAGGGTTCTCGAAAAAGGAAGCGTACGGGTGCGATTTG-  
-ATTCCTTTTCTCTTTTGGTTAAATCGTTGTTGATTTATTATTTGCTATATTTATTTTTC-  
-TAAGAAAAAGAGAAAACCAATTTAGCAACAACTAAATAATAAACGATATAAATAAAAAG-  
40 -GATAATTATCAACTAGAGAAGGAACAATTAATGGTATGTTTCATACACGCATGTAAAAATA-  
-CTATTAATAGTTGATCTCTTCTTGTAAATACCATAACAAGTATGTGCGTACATTTTAT-  
-AACTATCTATATAGTTGTCTTTCTCTGAATGTGCAAACTAAGCATTCGGAAGCCATTAT-  
-TTGATAGATATATCAACAGAAAGAGACTTACACGTTTTGATTCGTAAGGCTTCGGTAATA-  
45 -TAGCAGTATGAATAGGGAACTAAACCCAGTGATAAGACCTGATGATTTTCGCTTCTTTAA-  
-ATCGTCATACTTATCCCTTTGATTTGGGTCATTTCTGGACTACTAAAGCGAAGAAATT-  
-TTACATTTGGAGATTTTTTATTTACAGCATTTGTTTTCAAATATATTCCAATTAATCGGTG-  
50 -AATGTAAACCTCTAAAAAATAAATGTCGTAACAAAAGTTTATATAAGGTTAATTAGCCAC-  
-AATGATTGGAGTTAGAATAATCTACTATAGGATCATATTTTATTAAATTAGCGTCATCAT-  
-TTACTAACCTCAATCTTATTAGATGATATCCTAGTATAAAATAATTTAATCGCAGTAGTA-  
55 -AATATTGCCTCCATTTTTTAGGGTAATTATCCAGAATTGAAATATCAGATTTAACCATAG-  
-TTATAACGGAGGTAAAAAATCCCATTAATAGGTCTTAACCTTTATAGTCTAAATTGGTATC-  
-AATGAGGATAAATGATCGCGAGTAAATAATATTCACAATGTACCATTTTAGTCATATCAG-



35 During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

The expression plasmid pAMG22-His can be derived from the Amgen expression vector pAMG22 by substituting the small DNA sequence between the unique NdeI ( #4795) and EcoRI ( #4818) restriction sites of pAMG22 with the following oligonucleotide duplex:

NdeI
NheI
EcoRI

5' TATGAAACATCATCACCATCACCATCATGCTAGCGTTAACGCGTTGG 3'

(SEQ ID NO:51)

50 3' ACTTTGTAGTAGTGGTAGTGGTAGTACGATCGCAATTGCGCAACCTTAA 5'

(SEQ ID NO:52)

MetLysHisHisHisHisHisHisHisAlaSerValAsnAlaLeuGlu  
(SEQ ID NO:168)

pAMG22

- 5 The expression plasmid pAMG22 can be derived from the  
Amgen expression vector pCFM1656 (ATCC #69576) which in  
turn be derived from the Amgen expression vector system  
described in US Patent No. 4,710,473 granted December  
1, 1987. The pCFM1656 plasmid can be derived from the  
10 described pCFM836 plasmid (Patent No. 4,710,473) by:  
(a) destroying the two endogenous NdeI restriction  
sites by end filling with T4 polymerase enzyme followed  
by blunt end ligation; (b) replacing the DNA sequence  
between the unique AatII and ClaI restriction sites  
15 containing the synthetic PL promoter with a similar  
fragment obtained from pCFM636 (patent No. 4,710,473)  
containing the PL promoter

AatII

- 20 5' CTAATTCGCTCTCACCTACCAAACAATGCCCCCTGCAAAAATAAATTCATAT-  
3'TGCAGATTAAGGCGAGAGTGGATGGTTTGTACGGGGGACGTTTTTTATTTAAGTATA-  
  
-AAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-  
-TTTTTTGTATGTCTATTGGTAGACGCCACTATTTAATAGAGACCGCCACAACGTATTT-  
25 -TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)  
-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

ClaI

- 30 and then (c) substituting the small DNA sequence  
between the unique ClaI and KpnI restriction sites  
with the following oligonucleotide:

- 5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC 3'  
35 (SEQ ID NO:55)

3' TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC 5'  
(SEQ ID NO:56)

ClaI

KpnI

5 The expression plasmid pAMG22 can then be derived from  
pCFM1656 by making a series of site directed base  
changes by PCR overlapping oligo mutagenesis and DNA  
sequence substitutions. Starting with the BglIII site  
(plasmid bp # 180) immediately 5' to the plasmid  
10 replication promoter PcopB and proceeding toward the  
plasmid replication genes, the base pair changes are as  
follows:

15	<u>pAMG22 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG22</u>
	# 204	T/A	C/G
	# 428	A/T	G/C
20	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
	# 679	G/C	T/A
	# 980	T/A	C/G
25	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
	# 1028	A/T	T/A
	# 1047	C/G	T/A
30	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
	# 2480	A/T	T/A
35	# 2499-2502	AGTG TCAC	GTCA CAGT
40	# 2642	TCCGAGC AGGCTCG	7 bp deletion
	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A
45			

The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

5

[AatII sticky end] (position #4358 in pAMG22)

```

5'      GCGTAACGTATGCATGGTCTCCCATGCGAGAGTAGGGAAGTCCAGGCATCAA-
3' TGCACGCATTGCATACGTACCAGAGGGGTACGCTCTCATCCCTTGACGGTCCGTAGTT-
10 -ATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCCTTCGTTTTATCTGTTGTTTGTGCGGTG-
   -TATTTTGCTTTCCGAGTCAGCTTTCTGACCCGAAAGCAAATAGACAACAAACAGCCAC-

15 -AACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGG-
   -TTGCGAGAGGACTCATCTGTTTAGGCGGCCCTCGCCTAAACTTGCAACGCTTCGTTGCC-

   -CCCGGAGGGTGGCGGGCAGGACGCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAG-
   -GGGCCTCCACCGCCCGTCTGCGGGCGGTATTTGACGGTCCGTAGTTTAATTCGTCTTC-
20 -GCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTTTGTATTATTTTCTAAAT-
   -CGGTAGGACTGCCTACCGGAAAAACGCAAAGATGTTTGAGAAAACAAATAAAAAGATTTA-

      AatII
25 -ACATTCAAATATGGACGTCTCATAATTTTTAAAAAATTCATTTGACAAATGCTAAAATTC-
   -TGTAAGTTTATACCTGCAGAGTATTAAAAATTTTTTAAGTAAACTGTTTACGATTTTAAG-

   -TTGATTAATATTCTCAATTGTGAGCGCTCACAATTTATCGATTTGATTCTAGATTTGTTT-
   -AACTAATTATAAGAGTTAACTACTCGCGAGTGTTAAATAGCTAAACTAAGATCTAACTCA-
30 -TAACTAATTAAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGAGCTCACTAGTGT-
   -ATTGATTAATTTCTCTTATTGTATACCAATTGCGCAACCTTAAGCTCGAGTGATCACA-

      SacII
35 -CGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCGAAAGAAGAAGAAGAA-
   -GCTGGACGTCCCATGGTACCTTCGAATGAGCTCCTAGGCGCCTTCTTCTTCTTCTT-

   -GAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC-
   -CTTTCGGGCTTTCTTCGACTCAACCGACGACGGTGGCGACTCGTTATTGATCGTATTGG-
40 -CCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTGTCTGAAAGGAGGAACCGCTCTTCA-
   -GGAACCCCGGAGATTGCCCAGAACTCCCCAAAAACGACTTTCCTCCTTGCGGAGAAGT-

   -CGCTCTTCACGC 3' (SEQ ID NO:58)
45 -GCGAGAAGTG 5' (SEQ ID NO:57)

```

[SacII sticky end] (position #5024 in pAMG22)

During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

B. Human OPG Met[32-401]

In the example, the expression vector used was pAMG21, a derivative of pCFM1656 (ATCC accession no. 69576) which contains appropriate restriction sites for insertion of genes downstream from the lux PR promoter. (See U.S. Patent No. 5,169,318 for description of the lux expression system). The host cell used was GM120 (ATCC accession no. 55764). This host has the lacIQ promoter and lacI gene integrated into a second site in the host chromosome of a prototrophic E. coli K12 host. Other commonly used E. coli expression vectors and host cells are also suitable for expression.

A DNA sequence coding for an N-terminal methionine and amino acids 32-401 of the human OPG polypeptide was placed under control of the luxPR promoter in the plasmid expression vector pAMG21 as follows. To accomplish this, PCR using oligonucleotides #1257-20 and #1257-19 as primers was performed using as a template plasmid pRCCMV-Hu OPG DNA containing the human OPG cDNA and thermocycling for 30 cycles with each cycle being: 94°C for 20 seconds, followed by 37°C for 30 seconds, followed by 72°C for 30 seconds. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, and restricted with KpnI and BamHI restriction endonucleases and purified. Synthetic oligonucleotides #1257-21 and #1257-22 were phosphorylated individually using T4 polynucleotide kinase and ATP, and were then mixed together, heated at 94°C and allowed to slow cool to room temperature to form an oligonucleotide linker duplex containing NdeI and KpnI sticky ends. The phosphorylated linker duplex formed between oligonucleotides #1257-21 and #1257-22 containing NdeI and KpnI cohesive ends (see Figure 14A) and the KpnI and BamHI digested and purified PCR product generated

using oligo primers #1257-20 and #1257-19 (see above)  
was directionally inserted between two sites of the  
plasmid vector pAMG21, namely the NdeI site and BamHI  
site, using standard recombinant DNA methodology (see  
5 Figure 14A and sequences below). The synthetic linker  
utilized E. coli codons and provided for a N-terminal  
methionine.

Two clones were selected and plasmid DNA  
isolated, and the human OPG insert was subsequently DNA  
10 sequence confirmed. The resulting pAMG21 plasmid  
containing amino acids 32-401 of the human OPG  
polypeptide immediately preceded in frame by a  
methionine is referred to as pAMG21-huOPG met[32-401]  
or pAMG21-huOPG met[32-401].

15

Oligo#1257-19

5'-TACGCACTGGATCCTTATAAGCAGCTTATTTTTACTGATTGGAC-3'

(SEQ ID NO:59)

20

Oligo#1257-20

5'-GTCCTCCTGGTACCTACCTAAAACAAC-3' (SEQ ID NO:60)

Oligo#1257-21

5'-TATGGATGAAGAACTTCTCATCAGCTGCTGTGTGATAAATGTCC

25

GCCGGGTAC -3' (SEQ ID NO:61)

Oligo#1257-22

5'-CCGGCGGACATTTATCACACAGCAGCTGATGAGAAGTTTCTTCATCCA-3'

(SEQ ID NO:47)

30

Cultures of pAMG21-huOPG met[32-401] in E.  
coli GM120 in 2XYT media containing 20 µg/ml kanamycin  
were incubated at 30°C prior to induction. Induction  
of huOPG met[32-401] gene product expression from the  
35 luxPR promoter was achieved following the addition of  
the synthetic autoinducer N-(3-oxohexanoyl)-DL-

homoserine lactone to the culture media to a final concentration of 30 ng/ml and cultures were incubated at either 30°C or 37°C for a further 6 hours. After 6 hours, the bacterial cultures were examined by

5 microscopy for the presence of inclusion bodies and were then pelleted by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that some of the recombinant huOPG met[32-401] gene product was produced insolubly in *E. coli*.

10 Some bacterial pellets were resuspended in 10mM Tris-HCl/pH8, 1mM EDTA and lysed directly by addition of 2X Laemlli sample buffer to 1X final, and  $\beta$ -mercaptoethanol to 5% final concentration, and analyzed by SDS-PAGE. A substantially more intense coomassie

15 stained band of approximately 42kDa was observed on a SDS-PAGE gel containing total cell lysates of 30°C and 37°C induced cultures versus lane 2 which is a total cell lysate of a 30°C uninduced culture (Figure 14B). The expected gene product would be 370 amino acids in

20 length and have an expected molecular weight of about 42.2 kDa. Following induction at 37°C for 6 hours, an additional culture was pelleted and either processed for isolation of inclusion bodies (see below) or processed by microfluidizing. The pellet processed for

25 microfluidizing was resuspended in 25mM Tris-HCl/pH8, 0.5M NaCl buffer and passed 20 times through a Microfluidizer Model 1108 (Microfluidics Corp.) and collected. An aliquot was removed of the collected sample (microfluidized total lysate), and the remainder

30 was pelleted at 20,000 x g for 20 minutes. The supernatant following centrifugation was removed (microfluidized soluble fraction) and the pellet resuspended in a 25mM Tris-HCl/pH8, 0.5M NaCl, 6M urea solution (microfluidized insoluble fraction). To an

35 aliquot of either the total soluble, or insoluble fraction was added to an equal volume of 2X Laemalli

sample buffer and  $\beta$ -mercaptoethanol to 5% final concentration. The samples were then analyzed by SDS-PAGE. A significant amount of recombinant huOPG met[32-401] gene product appeared to be found in the insoluble fraction. To purify the recombinant protein inclusion bodies were purified as follows: Bacterial cells were separated from media by density gradient centrifugation in a Beckman J-6B centrifuge equipped with a JS-4.2 rotor at 4,900 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 5 ml of water and then diluted to a final volume of 10 ml with water. This suspension was transferred to a stainless steel cup cooled in ice and subjected to sonic disruption using a Branson Sonifier equipped with a standard tip (power setting=5, duty cycle=95%, 80 bursts). The sonicated cell suspension was centrifuged in a Beckman Optima TLX ultracentrifuge equipped with a TLA 100.3 rotor at 195,000 x g for 5 to 10 minutes at 23°C. The supernatant was discarded and the pellet rinsed with a stream of water from a squirt bottle. The pellets were collected by scraping with a micro spatula and transferred to a glass homogenizer (15 ml capacity). Five ml of Percoll solution (75% liquid Percoll, 0.15 M sodium chloride) was added to the homogenizer and the contents are homogenized until uniformly suspended. The volume was increased to 19.5 ml by the addition of Percoll solution, mixed, and distributed into 3 Beckman Quick-Seal tubes (13 x 32 mm). Tubes were sealed according to manufacturers instructions. The tubes were spun in a Beckman TLA 100.3 rotor at 23°C, 20,000 rpm (21,600 x g), 30 minutes. The tubes were examined for the appropriate banding pattern. To recover the refractile bodies, gradient fractions were recovered and pooled, then diluted with water. The inclusion



bodies were pelleted by centrifugation, and the protein concentration estimated following SDS-PAGE.

An aliquot of inclusion bodies isolated as described below was dissolved into 1X Laemlli sample  
5 buffer with 5%  $\beta$ -mercaptoethanol and resolved on a SDS-PAGE gel and the isolated inclusion bodies provide a highly purified recombinant huOPG[32-401] gene product. The major ~42 kDa band observed after resolving  
10 inclusion bodies on a SDS-polyacrylamide gel was excised from a separate gel and the N-terminal amino acid sequence determined essentially as described (Matsudaira et al. J. Biol. Chem. 262, 10-35 (1987)). The following sequence was determined after 19 cycles:

15       NH<sub>2</sub> -MDEETSHQLLCDKCPPGTY-COOH (SEQ ID NO:62)

This sequence was found to be identical to the first 19 amino acids encoded by the pAMG21 Hu-OPG met[32-401] expression vector, produced by a methionine residue  
20 provided by the bacterial expression vector.

C. Human OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG  
25 was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. Isolated plasmid DNA of pAMG21-huOPG met[32-401] (see Section B) was cleaved with KpnI and BamHI restriction endonucleases and the resulting fragments  
30 were resolved on an agarose gel. The B fragment (~1064 bp fragment) was isolated from the gel using standard methodology. Synthetic oligonucleotides (oligos) #1267-06 and #1267-07 were phosphorylated individually and allowed to form an oligo linker duplex, which  
35 contained NdeI and KpnI cohesive ends, using methods described in Section B. The synthetic linker duplex

utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated oligo linker containing NdeI and KpnI cohesive ends and the isolated ~1064 bp fragment of pAMG21-huOP met[32-401] digested with KpnI and BamHI restriction endonucleases were directionally inserted between the NdeI and BamHI sites of pAMG21 using standard recombinant DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401] gene.

Oligo #1267-06

5'-TAT GGA AAC TTT TCC TCC AAA ATA TCT TCA TTA TGA TGA  
AGA AAC TTC TCA TCA GCT GCT GTG TGA TAA ATG TCC GCC GGG  
TAC-3' (SEQ ID NO:63)

Oligo #1267-07

5'-CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAG AAG TTT  
CTT CAT CAT AAT GAA GAT ATT TTG GAG GAA AAG TTT CCA-3'  
(SEQ ID NO:64)

Cultures of pAMG21-huOPG-met[22-401] in E. coli host 393 were placed in 2XYT media containing 20 µg/ml kanamycin and were incubated at 30°C prior to induction. Induction of recombinant gene product expression from the luxPR promoter of vector pAMG21 was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and incubation at either 30°C or 37°C for a further 6 hours. After 6 hours, bacterial cultures were pelleted by centrifugation (=30°C I+6 or 37°C I+6). Bacterial cultures were also either pelleted just prior to induction (=30°C PreI) or alternatively no autoinducer

was added to a separate culture which was allowed to incubate at 30°C for a further 6 hours to give an uninduced (UI) culture (=30°C UI). Bacterial pellets of either 30°C PreI, 30°C UI, 30°C I+6, or 37°C I+6 cultures were resuspended, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Section B. Polyacrylamide gels were either stained with coomassie blue and/or Western transferred to nitrocellulose and immunoprobed with rabbit anti-mu OPG-Fc polyclonal antibody as described in Example 10. The level of gene product following induction compared to either an uninduced (30°C UI) or pre-induction (30°C PreI) sample.

15 D. Murine OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of the murine (mu) OPG (OPG) polypeptide was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1257-16 and #1257-15 as primers, plasmid pRCCMV-Mu OPG DNA as a template and thermocycling conditions as described in Section B. The PCR product was purified and cleaved with KpnI and BamHI restriction endonucleases as described in Section B. Synthetic oligos #1260-61 and #1260-82 were phosphorylated individually and allowed to form an oligo linker duplex with NdeI and KpnI cohesive ends using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1260-61 and #1260-82 containing NdeI and KpnI cohesive ends and the KpnI and BamHI digested and purified PCR product generated using oligo primers #1257-16 and #1257-15 were directionally inserted between the NdeI and BamHI sites of pAMG21

using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the MuOPG met[22-401] gene.

Expression of recombinant muOPG met[22-401] polypeptide from cultures of 393 cells harboring plasmid pAMG21-MuOPG met[22-401] following induction was determined using methods described in Section C.

Oligo #1257-15

5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTC ACG  
GAT TGA AC-3' (SEQ ID NO:65)

Oligo #1257-16

5'-GTG CTC CTG GTA CCT ACC TAA AAC AGC ACT GCA CAG  
TG-3' (SEQ ID NO:66)

Oligo #1260-61

5'-TAT GGA AAC TCT GCC TCC AAA ATA CCT GCA TTA CGA  
TCC GGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC  
GGG TAC-3' (SEQ ID NO:67)

Oligo #1260-82

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG  
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT  
CCA-3' (SEQ ID NO:68)

E. Murine OPG met[32-401]

A DNA sequence coding for an N-terminal methionine and amino acids 32 through 401 of murine OPG was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. To accomplish this, Synthetic oligos #1267-08 and #1267-09 were phosphorylated individually and

allowed to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed  
5 between oligos #1267-08 and #1267-09 containing NdeI and KpnI cohesive ends, and the KpnI and BamHI digested and purified PCR product described earlier (see Section D), was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The  
10 ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[32-401] gene.

15 Expression of recombinant muOPG-met [32-401] polypeptide from cultures of 393 cells harboring the pAMG21 recombinant plasmid following induction was determined using methods described in Section C.

20 Oligo #1267-08

5'-TAT GGA CCC AGA AAC TGG TCA TCA GCT GCT GTG TGA  
TAA ATG TGC TCC GGG TAC-3' (SEQ ID NO:69)

Oligo #1267-09

25 5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG  
TTT CTG GGT CCA-3' (SEQ ID NO:70)

#### F. Murine OPG met-lys[22-401]

A DNA sequence coding for an N-terminal  
30 methionine followed by a lysine residue and amino acids 22 through 401 of murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. Synthetic oligos #1282-95 and #1282-96 were phosphorylated individually and allowed  
35 to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E.

coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1282-95 and #1282-96 containing NdeI and KpnI cohesive ends and the KpnI and BamHI digested and purified PCR product described in Section D was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the MuOPG--Met-Lys[22-401] gene.

Expression of recombinant MuOPG Met-Lys[22-401] polypeptide from transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1282-95

5'-TAT GAA AGA AAC TCT GCC TCC AAA ATA CCT GCA TTA  
CGA TCC GGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC  
TCC GGG TAC-3' (SEQ ID NO:71)

Oligo #1282-96

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG  
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT  
TCA-3' (SEQ ID NO:72)

G. Murine OPG met-lys-(his)<sub>7</sub>[22-401]

A DNA sequence coding for N-terminal residues Met-Lys-His-His-His-His-His-His (=MKH) followed by amino acids 22 through 401 of Murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1300-50 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section

B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases and purified. The NdeI and BamHI digested and purified PCR product generated using oligo primers #1300-50 and #1257-15 was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing performed to verify the DNA sequence of the muOPG-MKH[22-401] gene.

Expression of recombinant MuOPG-MKH[22-401] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1300-50

5'-GTT CTC CTC ATA TGA AAC ATC ATC ACC ATC ACC ATC  
ATG AAA CTC TGC CTC CAA AAT ACC TGC ATT ACG AT-3' (SEQ  
ID NO:73)

Oligo #1257-15

(see Section D)

#### H. Murine OPG met-lys[22-401](his)<sub>7</sub>

A DNA sequence coding for a N-terminal met-lys, amino acids 22 through 401 murine OPG, and seven histidine residues following amino acid 401 (=muOPG MK[22-401]-H<sub>7</sub>), was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1300-49 and #1300-51 as primers and pAMG21-muOPG met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product

was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation was transformed into *E. coli* host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG MK[22-401]-H7 gene.

Expression of the recombinant muOPG MK-[22-401]-H7 polypeptide from a transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1300-49

5'-GTT CTC CTC ATA TGA AAG AAA CTC TGC CTC CAA AAT ACC TGC A-3' (SEQ ID NO:74)

Oligo #1300-51

5'-TAC GCA CTG GAT CCT TAA TGA TGG TGA TGG TGA TGA TGT AAG CAG CTT ATT TTC ACG GAT TGA ACC TGA TTC CCT A-3' (SEQ ID NO:75)

#### I. Murine OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-74 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI



restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[27-401] gene.

Expression of recombinant muOPG-met[27-401] polypeptide from a transfected 393 culture harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo#1309-74

5'-GTT CTC CTC ATA TGA AAT ACC TGC ATT ACG ATC CGG  
AAA CTG GTC AT-3' (SEQ ID NO:76)

Oligo#1257-15

(See Section D)

#### J. Human OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of human OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-75 and #1309-76 as primers and plasmid pAMG21-huOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with AseI and BamHI restriction endonucleases, and purified. The AseI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation

mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[27-401] gene.

Expression of the recombinant huOPG-met[27-401] polypeptide following induction of from transfected 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.

Oligo #1309-75

5'-GTT CTC CTA TTA ATG AAA TAT CTT CAT TAT GAT GAA  
GAA ACT T-3' (SEQ ID NO:77)

Oligo #1309-76

5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTT ACT  
GAT T-3' (SEQ ID NO:78)

#### 20 K. Murine OPG met[22-180]

A DNA sequence coding for a N-terminal methionine and amino acids 22 through 180 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-72 and #1309-73 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were

selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[22-180] gene.

5 Expression of recombinant muOPG-met[22-180] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1309-72

10 5'-GTT CTC CTC ATA TGG AAA CTC TGC CTC CAA AAT ACC  
TGC A-3' (SEQ ID NO:79)

Oligo #1309-73

15 5'-TAC GCA CTG GAT CCT TAT GTT GCA TTT CCT TTC TGA  
ATT AGC A-3' (SEQ ID NO:80)

L. Murine OPG met[27-180]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 180 of murine OPG  
20 was placed under the control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-74 (see Section I) and #1309-73 (see Section K) as primers and plasmid pAMG21-muOPG met[22-401] DNA as template.  
25 Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product  
30 above was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was  
35 isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG met[27-180] gene.

Expression of recombinant muOPG met[27-180] polypeptide from cultures of transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in  
5 Section C.

M. Murine OPG met[22-189] and met[22-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 189, or 22  
10 through 194 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1337-92 and #1337-93 (=muOPG-189 linker) or #1333-57 and #1333-58 (=muOPG-194 linker)  
15 were phosphorylated individually and allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid DNA of pAMG21-muOPG-met[22-401] was cleaved with KpnI and BspEI restriction endonucleases and the resulting DNA fragments were  
20 resolved on an agarose gel. The ~413 bp B fragment was isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1337-92 and #1337-93 (muOPG-189 linker) or oligos #1333-57 and #1333-58 (muOPG-194 linker)  
25 containing BspEI and BamHI cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of pAMG21-muOPG met[22-401]  
30 using standard methodology. Each ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the  
35 muOPG-met[22-189] or muOPG-met[22-194] genes.

Expression of recombinant muOPG-met[22-189] and muOPG-met[22-194] polypeptides from recombinant pAMG21 plasmids transformed into 393 cells was determined using methods described in Section C.

5

Oligo #1337-92

5'-CCG GAA ACA GAT AAT GAG-3' (SEQ ID NO:81)

Oligo #1337-93

10 5'-GAT CCT CAT TAT CTG TTT-3' (SEQ ID NO:82)

Oligo #1333-57

5'-CCG GAA ACA GAG AAG CCA CGC AAA AGT AAG-3'  
(SEQ ID NO:83)

15

Oligo #1333-58

5'-GAT CCT TAC TTT TGC GTG GCT TCT CTG TTT-3'  
(SEQ ID NO:84)

20 N. Murine OPG met[27-189] and met[27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 189, or 27 through 194 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers either "muOPG-189 linker" or "muOPG-194 linker" (see Section M) containing BspEI and BamHI cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases were directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-muOPG-met[27-401] using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing

was performed to verify the DNA sequence of either the muOPG met[27-189] or muOPG met[27-194] genes.

Expression of recombinant muOPG met[27-189] and muOPG met[27-194] following induction of 393 cells  
5 harboring recombinant pAMG21 plasmids was determined using methods described in Section C.

O. Human OPG met[22-185], met[22-189], met[22-194]

A DNA sequence coding for a N-terminal  
10 methionine and either amino acids 22 through 185, 22 through 189, or 22 through 194 of the human OPG polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1331-  
15 87 and #1331-88 (=huOPG-185 linker), #1331-89 and #1331-90 (=huOPG-189 linker), or #1331-91 & #1331-92 (=huOPG-194 linker) were phosphorylated individually and each allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid  
20 DNA of pAMG21-huOPG-met[27-401] was restricted with KpnI and NdeI restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~407 bp B fragment was isolated using standard recombinant DNA methodology. The  
25 phosphorylated oligo linker duplexes formed between either oligos #1331-87 and #1331-88 (huOPG-185 linker), oligos #1331-89 and #1331-90 (huOPG-189 linker), or oligos #1331-91 and #1331-92 (huOPG-194 linker)[each linker contains NdeI and BamHI cohesive ends], and the  
30 isolated ~407 bp B fragment of plasmid pAMG21-huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-huOPG-met[22-401] using standard methodology. Each ligation  
35 was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol.

Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the huOPG-met[22-185], huOPG-met[22-189], or huOPG-met[22-194] genes.

5            Expression of recombinant huOPG-met[22-185], huOPG-met[22-189] or huOPG-met[22-194] in transformed 393 cells harboring recombinant pAMG21 plasmids following induction was determined using methods described in Section C.

10

    Oligo #1331-87

    5'-TAT GTT AAT GAG-3' (SEQ ID NO:85)

    Oligo #1331-88

15            5'-GAT CCT CAT TAA CA-3' (SEQ ID NO:86)

    Oligo #1331-89

    5'-TAT GTT CCG GAA ACA GTT AAG-3' (SEQ ID NO:87)

20

    Oligo #1331-90

    5'-GAT CCT TAA CTG TTT CCG GAA CA-3' (SEQ ID NO:88)

    Oligo #1331-91

25            5'-TAT GTT CCG GAA ACA GTG AAT CAA CTC AAA AAT AAG-3' (SEQ ID NO:89)

    Oligo #1331-92

30            5'-GAT CCT TAT TTT TGA GTT GAT TCA CTG TTT CCG GAA CA-3' (SEQ ID NO:90)

P. Human OPG met[27-185], met[27-189], met [27-194]

35            A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 185, 27 through 189, or 27 through 194 of the human OPG

polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers "huOPG-185 linker", "huOPG-189 linker", or "huOPG-194 linker" (See  
5 Section O) each containing NdeI and BamHI cohesive ends, and the isolated ~407 bp B fragment of plasmid pAMG21-huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases (See Section O) were  
10 directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-huOPG-met[27-401] (See Section J) using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA isolated, and DNA sequencing  
15 performed to verify the DNA sequence of either the huOPG-met[27-185], huOPG-met[27-189], or huOPG-met[27-194] genes.

Expression of recombinant huOPG-met[27-185], huOPG-met[27-189], and huOPG-met[27-194] from  
20 recombinant pAMG21 plasmids transformed into 393 cells was determined using methods described in Section C.

O. Murine OPG met[27-401] (P33E, G36S, A45P)

25 A DNA sequence coding for an N-terminal methionine and amino acids 27 through 48 of human OPG followed by amino acid residues 49 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as  
30 follows. Purified plasmid DNA of pAMG21-huOPG-met[27-401] (See Section J) was cleaved with AatII and KpnI restriction endonucleases and a ~1075 bp B fragment isolated from an agarose gel using standard recombinant DNA methodology. Additionally, plasmid pAMG21-muOPG-  
35 met[22-401] DNA (See Section D) was digested with KpnI and BamHI restriction endonucleases and the ~1064 bp B



fragment isolated as described above. The isolated  
~1075 bp pAMG21-huOPG-met[27-401] restriction fragment  
containing AatII & KpnI cohesive ends (see above), the  
~1064 bp pAMG21-muOPG-met[22-401] restriction fragment  
5 containing KpnI and BamHI sticky ends and a ~5043 bp  
restriction fragment containing AatII and BamHI  
cohesive ends and corresponding to the nucleic acid  
sequence of pAMG21 between AatII & BamHI were ligated  
using standard recombinant DNA methodology. The  
10 ligation was transformed into E. coli host 393 by  
electroporation utilizing the manufacturer's protocol.  
Clones were selected, and the presence of the  
recombinant insert in the plasmid verified using  
standard DNA methodology. muOPG-27-401 (P33E, G36S,  
15 A45P) gene. Amino acid changes in muOPG from proline-  
33 to glutamic acid-33, glycine-36 to serine-36, and  
alanine-45 to proline-45, result from replacement of  
muOPG residues 27 through 48 with huOPG residues 27  
through 48.

20 Expression of recombinant muOPG-met[27-401]  
(P33E, G36S, A45P) from transformed 393 cells harboring  
the recombinant pAMG21 plasmid was determined using  
methods described in Section C.

25 R. Murine OPG met-lys-(his)<sub>7</sub>-ala-ser-(asp)<sub>4</sub>-lys[22-401]  
(A45T)

A DNA sequence coding for an N-terminal His  
tag and enterokinase recognition sequence which is (NH<sub>2</sub>  
30 to COOH terminus): Met-Lys-His-His-His-His-His-  
His-Ala-Ser-Asp-Asp-Asp-Asp-Lys (=HEK), followed by  
amino acids 22 through 401 of the murine OPG  
polypeptide was placed under control of the lac  
repressor regulated Ps4 promoter as follows. pAMG22-  
35 His (See Section A) was digested with NheI and BamHI  
restriction endonucleases, and the large fragment (the

A fragment) isolated from an agarose gel using standard recombinant DNA methodology. Oligonucleotides #1282-91 and #1282-92 were phosphorylated individually and allowed to form an oligo linker duplex using methods previously described (See Section B). The phosphorylated linker duplex formed between oligos #1282-91 and #1282-92 containing NheI and KpnI cohesive ends, the KpnI and BamHI digested and purified PCR product described (see Section D), and the A fragment of vector pAMG22-His digested with NheI and BamHI were ligated using standard recombinant DNA methodology. The ligation was transformed into E. coli host GM120 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA isolated and DNA sequencing performed to verify the DNA sequence of the muOPG-HEK[22-401] gene. DNA sequencing revealed a spurious mutation in the natural muOPG sequence that resulted in a single amino acid change of Alanine-45 of muOPG polypeptide to a Threonine.

Expression of recombinant muOPG-HEK[22-401] (A45T) from GM120 cells harboring the recombinant pAMG21 plasmid was determined using methods similar to those described in Section C, except instead of addition of the synthetic autoinducer, IPTG was added to 0.4 mM final to achieve induction.

Oligo #1282-91

5'-CTA GCG ACG ACG ACG ACA AAG AAA CTC TGC CTC CAA  
AAT ACC TGC ATT ACG ATC CGG AAA CTG GTC ATC AGC TGC TGT  
GTG ATA AAT GTG CTC CGG GTA C-3' (SEQ ID NO:91)

Oligo #1282-92

5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG  
TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT CTT  
TGT CGT CGT CGT CG-3' (SEQ ID NO:92)

S. Human OPG met-arg-gly-ser-(his)<sub>6</sub>[22-401]

Eight oligonucleotides (1338-09 to 1338-16 shown below) were designed to produce a 175 base fragment as overlapping, double stranded DNA. The oligos were annealed, ligated, and the 5' and 3' oligos were used as PCR primers to produce large quantities of the 175 base fragment. The final PCR gene products were digested with restriction endonucleases ClaI and KpnI to yield a fragment which replaces the N-terminal 28 codons of human OPG. The ClaI and KpnI digested PCR product was inserted into pAMG21-huOPG [27-401] which had also been cleaved with ClaI and KpnI. Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Expression of huOPG Met-Arg-Gly-Ser-(His)<sub>6</sub> [22-401] resulting in the formation of large inclusion bodies and the protein was localized to the insoluble (pellet) fraction.

1338-09

ACA AAC ACA ATC GAT TTG ATA CTA GA (SEQ ID NO:93)

1338-10

TTT GTT TTA ACT AAT TAA AGG AGG AAT AAA ATA TGA GAG GAT CGC ATC AC  
(SEQ ID NO:94)

1338-11

CAT CAC CAT CAC GAA ACC TTC CCG CCG AAA TAC CTG CAC TAC GAC GAA.GA  
(SEQ ID NO:95)

1338-12

AAC CTC CCA CCA GCT GCT GTG CGA CAA ATG CCC GCC GGG TAC CCA AAC A  
(SEQ ID NO:96)

5

1338-13

TGT TTG GGT ACC CGG CGG GCA TTT GT (SEQ ID NO:97)

1338-14

10 CGC ACA GCA GCT GGT GGG AGG TTT CTT CGT CGT AGT GCA GGT ATT TCG GC  
(SEQ ID NO:98)

1338-15

15 GGG AAG GTT TCG TGA TGG TGA TGG TGA TGC GAT CCT CTC ATA TTT TAT T  
(SEQ ID NO:99)

1338-16

CCT CCT TTA ATT AGT TAA AAC AAA TCT AGT ATC AAA TCG ATT GTG TTT GT  
(SEQ ID NO:100)

20

T. Human OPG met-lys[22-401] and met-(lys)<sub>3</sub>[22-401]

To construct the met-lys and met-(lys)<sub>3</sub> versions of human OPG[22-401], overlapping oligonucleotides were designed to add the appropriate number of lysine residues. The two oligos for each construct were designed to overlap, allowing two rounds of PCR to produce the final product. The template for the first PCR reaction was a plasmid DNA preparation containing the human OPG 22-401 gene. The first PCR added the lysine residue(s). The second PCR used the product of the first round and added sequence back to the first restriction site, ClaI.

The final PCR gene products were digested with restriction endonucleases ClaI and KpnI, which replace the N-terminal 28 codons of hu OPG, and then ligated into plasmid pAMG21-hu OPG [27-401] which had

been also digested with the two restriction endonucleases. Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Neither construct had a detectable level of protein expression and inclusion bodies were not visible. The DNA sequences were confirmed by DNA sequencing.

Oligonucleotide primers to prepare Met-Lys huOPG[22-401]:  
1338-17  
ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT  
AAA GGA GGA ATA AAA TG (SEQ ID NO:101)

1338-18  
CTA ATT AAA GGA GGA ATA AAA TGA AAG AAA CTT TTC CTC CAA  
AAT ATC (SEQ ID NO:102)

1338-20  
TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID NO:103)

Oligonucleotide primers to prepare Met-(Lys)<sub>3</sub>-huOPG[22-401]:  
1338-17  
ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT  
AAA GGA GGA ATA AAA TG (SEQ ID NO:104)

35

1338-19

CTA ATT AAA GGA GGA ATA AAA TGA AAA AAA AAG AAA CTT TTC  
CTC CAA AAT ATC (SEQ ID NO:105)

5 1338-20

TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID  
NO:106)

U. Human and Murine OPG [22-401]/Fc Fusions

10 Four OPG-Fc fusions were constructed where  
the Fc region of human IgG1 was fused at the N-terminus  
of either human or murine Osteoprotegerin amino acids  
22 to 401 (referred to as Fc/OPG [22-401]) or at the C-  
terminus (referred to as OPG[22-401]/Fc). Fc fusions  
15 were constructed using the fusion vector pFc-A3  
described in Example 7.

All fusion genes were constructed using  
standard PCR technology. Template for PCR reactions  
were plasmid preparations containing the target genes.  
20 Overlapping oligos were designed to combine the  
C-terminal portion of one gene with the N terminal  
portion of the other gene. This process allows fusing  
the two genes together in the correct reading frame  
after the appropriate PCR reactions have been  
25 performed. Initially one "fusion" oligo for each gene  
was put into a PCR reaction with a universal primer for  
the vector carrying the target gene. The complimentary  
"fusion" oligo was used with a universal primer to PCR  
the other gene. At the end of this first PCR reaction,  
30 two separate products were obtained, with each  
individual gene having the fusion site present,  
creating enough overlap to drive the second round of  
PCR and create the desired fusion. In the second round  
of PCR, the first two PCR products were combined along  
35 with universal primers and via the overlapping regions,  
the full length fusion DNA sequence was produced.

The final PCR gene products were digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMG21 having been also digested with the two restriction endonucleases.

- 5 Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were  
10 determined from 50 ml shaker flask studies. Whole cell lysate, sonic pellet, and supernatant were analyzed for expression of the fusion by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody.

15 Fc/huOPG [22-401]

- Expression of the Fc/hu OPG [22-401] fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The cells have very large inclusion bodies, and the majority of the product is in  
20 the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc fusion:

1318-48

- 25 CAG CCC GGG TAA AAT GGA AAC GTT TCC TCC AAA ATA TCT TCA  
TT (SEQ ID NO:107)

1318-49

- CGT TTC CAT TTT ACC CGG GCT GAG CGA GAG GCT CTT CTG CGT  
30 GT (SEQ ID NO:108)

Fc/muOPG [22-401]

- Expression of the fusion peptide was detected on a Coomassie stained gel and on a Western blot. The  
35 cells have very large inclusion bodies, and the majority of the product is in the insoluble (pellet)

fraction. The following primers were used to construct this OPG-Fc fusion:

1318-50

5 CGC TCA GCC CGG GTA AAA TGG AAA CGT TGC CTC CAA AAT ACC  
TGC (SEQ ID NO:109)

1318-51

CCA TTT TAC CCG GGC TGA GCG AGA GGC TCT TCT GCG TGT  
10 (SEQ ID NO:110)

muOPG [22-401]/Fc

Expression of the fusion peptide was detected on a Coomassie stained gel and on a Western blot. The  
15 amount of recombinant product was less than the OPG  
fusion proteins having the Fc region in the N terminal  
position. Obvious inclusion bodies were not detected.  
Most of the product appeared to be in the insoluble  
(pellet) fraction. The following primers were used to  
20 construct this OPG-Fc fusion:

1318-54

GAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC  
(SEQ ID NO:111)

25

1318-55

CAG CTG CAG CTA AGC AGC TTA TTT TCA CGG ATT G  
(SEQ ID NO:112)

30

huOPG [22-401]/Fc

Expression of the fusion peptide was not detected on a Coomassie stained gel, although a faint  
Western positive signal was present. Obvious inclusion  
35 bodies were not detected. The following primers were  
used to prepare this OPG-Fc fusion:



1318-52

AAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC  
(SEQ ID NO:113)

5

1318-53

CAG CTG CAG CTA AGC AGC TTA TTT TTA CTG ATT GG  
(SEQ ID NO:114)

10 V. Human OPG met[22-401]-Fc fusion (P25A)

This construct combines a proline to alanine amino acid change at position 25 (P25A) with the huOPG met[22-401]-Fc fusion. The plasmid was digested with restriction endonucleases ClaI and KpnI, which removes  
15 the N-terminal 28 codons of the gene, and the resulting small (less than 200 base pair) fragment was gel purified. This fragment containing the proline to alanine change was then ligated into plasmid pAMG21-huOPG [22-401]-Fc fusion which had been digested with  
20 the two restriction endonucleases. The ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein  
25 expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. The expression level of the fusion  
30 peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The protein was in the insoluble (pellet) fraction. The cells had large inclusion bodies.

W. Human OPG met[22-401] (P25A)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the proline at position 25 being substituted by alanine under control of the lux Pr promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-84 and 1289-85 were annealed to form an oligo linker duplex with XbaI and KpnI cohesive ends. The synthetic linker duplex utilized optimal E. coli codons and encoded an N-terminal methionine. The linker also included an SpeI restriction site which was not present in the original sequence. The linker duplex was directionally inserted between the XbaI and KpnI sites in pAMG21-huOPG-22-401 using standard methods. The ligation mixture was introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the HuOPG-Met[22-401](P25A) gene. The following oligonucleotides were used to generate the XbaI - KpnI linker:

Oligo #1289-84

5'-CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TGC TCC  
AAA ATA TCT TCA TTA TGA TGA AGA AAC TAG TCA TCA GCT GCT  
GTG TGA TAA ATG TCC GCC GGG TAC -3' (SEQ ID NO:115)

Oligo #1289-85

5'- CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAC  
TAG TTT CTT CAT CAT AAT GAA GAT ATT TTG GAG CAA AAG TTT  
CCA TAT GTT ATT CCT CCT T-3' (SEQ ID NO:116)

X. Human OPG met[22-401] (P26A) and (P26D)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the proline at position 26 being substituted by alanine under control of the lux Pr promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-86 and 1289-87 were annealed to form an oligo linker duplex with XbaI and SpeI cohesive ends. The synthetic linker duplex utilized optimal E. coli codons and encoded an N-terminal methionine. The linker duplex was directionally inserted between the XbaI and SpeI sites in pAMG21-huOPG[22-401] (P25A) using standard methods. The ligation mixture was introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401] (P26A) gene. One of the clones sequenced was found to have the proline at position 26 substituted by aspartic acid rather than alanine, and this clone was designated huOPG-met[22-401] (P26D). The following oligonucleotides were used to generate the XbaI - SpeI linker:

Oligo #1289-86

5' - CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TCC  
TGC TAA ATA TCT TCA TTA TGA TGA AGA AA - 3'  
(SEQ ID NO:117)

Oligo #1289-87

5' - CTA GTT TCT TCA TCA TAA TGA AGA TAT TTA GCA  
GGA AAA GTT TCC ATA TGT TAT TCC TCC TT - 3'  
(SEQ ID NO:118)

Y. Human OPG met[22-194] (P25A)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 194 of human OPG with the proline at position 25 being substituted by alanine under control of the lux Pr promoter in prokaryotic expression vector pAMG21 was constructed as follows: The plasmids pAMG21-huOPG[27-194] and pAMG21-huOPG[22-401] (P25A) were each digested with KpnI and BamHI endonucleases. The 450 bp fragment was isolated from pAMG21-huOPG[27-194] and the 6.1 kbp fragment was isolated from pAMG21-huOPG[22-401] (P25A). These fragments were ligated together and introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the huOPG-Met[22-194] (P25A) gene.

EXAMPLE 9

Association of OPG Monomers

CHO cells engineered to overexpress muOPG [22-401] were used to generate conditioned media for the analysis of secreted recombinant OPG using rabbit polyclonal anti-OPG antibodies. An aliquot of conditioned media was concentrated 20-fold, then analysed by reducing and non-reducing SDS-PAGE (Figure 15). Under reducing conditions, the protein migrated as a Mr 50-55 kd polypeptide, as would be predicted if the mature product was glycosylated at one or more of its consensus N-linked glycosylation sites. Suprisingly, when the same samples were analysed by non-reducing SDS-PAGE, the majority of the protein migrated as an approximately 100 kd polypeptide, twice the size of the reduced protein. In addition, there

was a smaller amount of the Mr 50-55 kd polypeptide. This pattern of migration on SDS-PAGE was consistent with the notion that the OPG product was forming dimers through oxidation of a free sulfhydryl group(s).

5           The predicted mature OPG polypeptide contains 23 cysteine residues, 18 of which are predicted to be involved in forming intrachain disulfide bridges which comprise the four cysteine-rich domains (Figure 12A). The five remaining C-terminal cysteine residues are not  
10 involved in secondary structure which can be predicted based upon homology with other TNFR family members. Overall there is a net uneven number of cysteine residues, and it is formally possible that at least one residue is free to form an intermolecular disulfide  
15 bond between two OPG monomers.

To help elucidate patterns of OPG kinesis and monomer association, a pulse-chase labelling study was performed. CHO cells expressing muOPG [22-401] were metabolically labelled as described above in serum-free  
20 medium containing <sup>35</sup>S methionine and cysteine for 30 min. After this period, the media was removed, and replaced with complete medium containing unlabelled methionine and cysteine at levels approximately 2,000-fold excess to the original concentration of  
25 radioactive amino acids. At 30 min, 1hr, 2 hr, 4 hr, 6 hr and 12 hr post addition, cultures were harvested by the removal of the conditioned media, and lysates of the conditioned media and adherent monolayers were prepared. The culture media and cell lysates were  
30 clarified as described above, and then immunoprecipitated using anti-OPG antibodies as described above. After the immunoprecipitates were washed, they were released by boiling in non-reducing SDS-PAGE buffer then split into two equal halves. To  
35 one half, the reducing agent  $\beta$ -mercaptoethanol was added to 5% (v/v) final concentration, while the other half

was maintained in non-reducing conditions. Both sets of immunoprecipitates were analysed by SDS-PAGE as described above, then processed for autoradiography and exposed to film. The results are shown in Figure 16.

5 The samples analysed by reducing SDS-PAGE are depicted in the bottom two panels. After synthesis, the OPG polypeptide is rapidly processed to a slightly larger polypeptide, which probably represents modification by N-linked glycosylation. After approximately 1-2 hours,

10 the level of OPG in the cell decreases dramatically, and concomitantly appears in the culture supernatant. This appears to be the result of the vectorial transport of OPG from the cell into the media over time, consistent with the notion that OPG is a naturally

15 secreted protein. Analysis of the same immunoprecipitates under nonreducing conditions reveals the relationship between the formation of OPG dimers and secretion into the conditioned media (Figure 16, upper panels). In the first 30-60 minutes, OPG

20 monomers are processed in the cell by apparent glycosylation, followed by dimer formation. Over time, the bulk of OPG monomers are driven into dimers, which subsequently disappear from the cell. Beginning about 60 minutes after synthesis, OPG dimers appear in

25 the conditioned media, and accumulate over the duration of the experiment. Following this period, OPG dimers are formed, which are then secreted into the culture media. OPG monomers persist at a low level inside the cell over time, and small amounts also appear in the

30 media. This does not appear to be the result of breakdown of covalent OPG dimers, but rather the production of sub-stoichiometric amounts of monomers in the cell and subsequent secretion.

Recombinantly produced OPG from transfected

35 CHO cells appears to be predominantly a dimer. To determine if dimerization is a natural process in OPG

synthesis, we analysed the conditioned media of a cell line found to naturally express OPG. The CTLL-2 cell line, a murine cytotoxic T lymphocytic cell line (ATCC accession no. TIB-214), was found to express OPG mRNA in a screen of tissue and cell line RNA. The OPG transcript was found to be the same as the cloned and sequenced 2.5-3.0 kb RNA identified from kidney and found to encode a secreted molecule. Western blot analysis of conditioned media obtained from CTLL-2 cells shows that most, if not all, of the OPG protein secreted is a dimer (Figure 17). This suggests that OPG dimerization and secretion is not an artifact of overexpression in a cell line, but is likely to be the main form of the product as it is produced by expressing cells.

Normal and transgenic mouse tissues and serum were analysed to determine the nature of the OPG molecule expressed in OPG transgenic mice. Since the rat OPG cDNA was expressed under the control of a hepatocyte control element, extracts made from the parenchyma of control and transgenic mice under non-reducing conditions were analysed (Figure 18). In extract from transgenic, but not control mice, OPG dimers are readily detected, along with substoichiometric amounts of monomers. The OPG dimers and monomers appear identical to the recombinant murine protein expressed in the genetically engineered CHO cells. This strongly suggests that OPG dimers are indeed a natural form of the gene product, and are likely to be key active components. Serum samples obtained from control and transgenic mice were similarly analysed by western blot analysis. In control mice, the majority of OPG protein migrates as a dimer, while small amounts of monomer are also detected. In addition, significant amounts of a larger OPG related protein is detected, which migrates with a

relative molecular mass consistent with the predicted size of a covalently-linked trimer. Thus, recombinant OPG is expressed predominantly as a dimeric protein in OPG transgenic mice, and the dimer form may be the basis for the osteopetrotic phenotype in OPG mice. OPG recombinant protein may also exist in higher molecular weight "trimeric" forms.

To determine if the five C-terminal cysteine residues of OPG play a role in homodimerization, the murine OPG codons for cysteine residues 195 (C195), C202, C277, C319, and C400 were changed to serine using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) as described above. The muOPG gene was subcloned between the Not I and Xba I sites of the pcDNA 3.1 (+) vector (Invitrogen, San Diego, CA). The resulting plasmid, pcDNA3.1-muOPG, and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent E. coli XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to change the codon for cysteine residue 195 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C195S protein:

1389-19:

5' -CAC GCA AAA GTC GGG AAT AGA TGT CAC-3'  
(SEQ ID NO:150)

1406-38:

5' -GTG ACA TCT ATT CCC GAC TTT TGC GTG-3'  
(SEQ ID NO:151)



The following primer pairs were used to change the codon for cysteine residue 202 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C202S protein:

1389-21:

5' -CAC CCT GTC GGA AGA GGC CTT CTT C-3'  
(SEQ ID NO:152)

1389-22:

5' -GAA GAA GGC CTC TTC CGA CAG GGT G-3' (1389-22)  
(SEQ ID NO:153)

The following primer pairs were used to change the codon for cysteine residue 277 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C277S protein:

1389-23:

5' -TGA CCT CTC GGA AAG CAG CGT GCA-3'  
(SEQ ID NO:154)

1389-24:

5' -TGC ACG CTG CTT TCC GAG AGG TCA-3'  
(SEQ ID NO:155)

The following primer pairs were used to change the codon for cysteine residue 319 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C319S protein:

1389-17:

5' -CCT CGA AAT CGA GCG AGC AGC TCC-3'  
(SEQ ID NO:156)

1389-18:

5' -CGA TTT CGA GGT CTT TCT CGT TCT C-3'  
(SEQ ID NO:157)

5

The following primer pairs were used to change the codon for cysteine residue 400 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C400S protein:

10

1406-72:

5' -CCG TGA AAA TAA GCT CGT TAT AAC TAG GAA TGG-3'  
(SEQ ID NO:158)

15 1406-75:

5' -CCA TTC CTA GTT ATA ACG AGC TTA TTT TCA CGG-3'  
(SEQ ID NO:159)

Each resulting muOPG [22-401] plasmid containing the appropriate mutation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in example 11. Conditioned media from each transfectant was analysed by non-reducing SDS-PAGE and western blotting with anti-OPG antibodies.

Mutation of any of the five C-terminal cysteine residues results in the production of predominantly (>90%) monomeric 55 kd OPG molecules. This strongly suggests that the C-terminal cysteine residues together play a role in OPG homodimerization.

C-terminal OPG deletion mutants were constructed to map the region(s) of the OPG C-terminal domain which are important for OPG homodimerization. These OPG mutants were constructed by PCR amplification

using primers which introduce premature stop translation signals in the C-terminal region of murine OPG. The 5' oligo was designed to the MuOPG start codon (containing a HindIII restriction site) and the 3' oligonucleotides (containing a stop codon and XhoI site) were designed to truncate the C-terminal region of muOPG ending at either threonine residue 200 (CT 200), proline 212 (CT212), glutamic acid 293 (CT-293), or serine 355 (CT-355).

10           The following primers were used to construct muOPG [22-200]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA  
15 ACA AG-3' (SEQ ID NO:160)

1391-91:

5' -CCT CTC TCG AGT CAG GTG ACA TCT ATT CCA CAC  
20 TTT TGC GTG GC-3' (1391-91) (SEQ ID NO:161)

20           The following primers were used to construct muOPG [22-212]:

1091-39:

25           5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA  
ACA AG-3' (SEQ ID NO:162)

1391-90:

5' -CCT CTC TCG AGT CAA GGA ACA GCA AAC CTG AAG  
30 AAG GC -3' (SEQ ID NO:163)

30           The following primers were used to construct muOPG [22-293]:

1091-39:

35           5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA  
ACA AG-3' (SEQ ID NO:164)

1391-89:

5' - CCT CTC TCG AGT CAC TCT GTG GTG AGG TTC GAG  
TGG CC-3' (SEQ ID NO:165)

5

The following primers were used to construct muOPG  
[22-355]:

1091-39:

10 5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA  
ACA AG-3' (SEQ ID NO:166)

1391-88:

15 5' CCT CTC TCG AGT CAG GAT GTT TTC AAG TGC TTG AGG GC-  
3'  
(SEQ ID NO:167)

Each resulting muOPG-CT plasmid containing  
the appropriate truncation was then transfected into  
20 human 293 cells, the mutant OPG-Fc fusion protein  
purified from conditioned media as described above.  
The biological activity of each protein was assessed  
the in vitro osteoclast forming assay described in  
example 11. The conditioned medias were also analysed  
25 by non-reducing SDS-PAGE and western blotting using  
anti-OPG antibodies.

Truncation of the C-terminal region of OPG  
effects the ability of OPG to form homodimers. CT 355  
is predominantly monomeric, although some dimer is  
30 formed. CT 293 forms what appears to be equal molar  
amounts of monomer and dimer, and also high molecular  
weight aggregates. However, CT 212 and CT 200 are  
monomeric.

EXAMPLE 10

Purification of OPG

A. Purification of mammalian OPG-Fc Fusion Proteins

5           5 L of conditioned media from 293 cells  
expressing an OPG-Fc fusion protein were prepared as  
follows. A frozen sample of cells was thawed into 10  
ml of 293S media (DMEM-high glucose, 1x L-glutamine,  
10% heat inactivated fetal bovine serum (FBS) and 100  
10 ug/ml hygromycin) and fed with fresh media after one  
day. After three days, cells were split into two T175  
flasks at 1:10 and 1:20 dilutions. Two additional 1:10  
splits were done to scale up to 200 T175 flasks. Cells  
were at 5 days post-thawing at this point. Cells were  
15 grown to near confluency (about three days) at which  
time serum-containing media was aspirated, cells were  
washed one time with 25 ml PBS per flask and 25 ml of  
SF media (DMEM-high glucose, 1x L-glutamine) was added  
to each flask. Cells were maintained at 5% CO<sub>2</sub> for  
20 three days at which point the media was harvested,  
centrifuged, and filtered through 0.45m cellulose  
nitrate filters (Corning).

OPG-Fc fusion proteins were purified using a  
Protein G Sepharose column (Pharmacia) equilibrated in  
25 PBS. The column size varied depending on volume of  
starting media. Conditioned media prepared as  
described above was loaded onto the column, the column  
washed with PBS, and pure protein eluted using 100mM  
glycine pH 2.7. Fractions were collected into tubes  
30 containing 1M Tris pH 9.2 in order to neutralize as  
quickly as possible. Protein containing fractions were  
pooled, concentrated in either an Amicon Centricon 10  
or Centriprep 10 and diafiltered into PBS. The pure  
protein is stored at -80°C.

35           Murine [22-401]-Fc, Murine [22-180]-Fc,  
Murine [22-194]-Fc, human [22-401]-Fc and human [22-

201]Fc were purified by this procedure. Murine [22-185]-Fc is purified by this procedure.

B. Preparation of anti-OPG antibodies

5           Three New Zealand White rabbits (5-8 lbs initial wt) were injected subcutaneously with muOPG[22-401]-Fc fusion protein. Each rabbit was immunized on day 1 with 50 µg of antigen emulsified in an equal volume of Freund's complete adjuvant. Further boosts  
10 (Days 14 and 28) were performed by the same procedure with the substitution of Freund's incomplete adjuvant. Antibody titers were monitored by EIA. After the second boost, the antisera revealed high antibody titers and 25ml production bleeds were obtained from each animal.  
15 The sera was first passed over an affinity column to which murine OPG-Fc had been immobilized. The anti-OPG antibodies were eluted with Pierce Gentle Elution Buffer containing 1% glacial acetic acid. The eluted protein was then dialyzed into PBS and passed over a Fc  
20 column to remove any antibodies specific for the Fc portion of the OPG fusion protein. The run through fractions containing anti-OPG specific antibodies were dialyzed into PBS.

25 C. Purification of murine OPG[22-401]

Antibody Affinity Chromatography

Affinity purified anti-OPG antibodies were diafiltered into coupling buffer (0.1M sodium carbonate  
30 pH 8.3, 0.5M NaCl), and mixed with CNBr-activated sepharose beads (Pharmacia) for two hours at room temperature. The resin was then washed with coupling buffer extensively before blocking unoccupied sites with 1M ethanolamine (pH 8.0) for two hours at room  
35 temperature. The resin was then washed with low pH (0.1M sodium acetate pH 4.0, 0.5M NaCl) followed by a

high pH wash (0.1M Tris-HCl pH 8.0, 0.5M NaCl). The last washes were repeated three times. The resin was finally equilibrated with PBS before packing into a column. Once packed, the resin was washed with PBS. A  
5 blank elution was performed with 0.1M glycine-HCl, pH 2.5), followed by re-equilibration with PBS.

Concentrated conditioned media from CHO cells expressing muOPG[22-410] was applied to the column at a low flow rate. The column was washed with PBS until UV  
10 absorbance measured at 280nm returned to baseline. The protein was eluted from the column first with 0.1M glycine-HCl (pH 2.5), re-equilibrated with PBS, and eluted with a second buffer (0.1M CAPS, pH 10.5), 1M NaCl). The two elution pools were diafiltered  
15 separately into PBS and sterile filtered before freezing at -20°C.

#### Conventional Chromatography

CHO cell conditioned media was concentrated  
20 23x in an Amicon spiral wound cartridge (S10Y10) and diafiltered into 20mM tris pH 8.0. The diafiltered media was then applied to a Q-sepharose HP (Pharmacia) column which had been equilibrated with 20mM tris pH 8.0. The column was then washed until absorbance at  
25 280nm reached baseline. Protein was eluted with a 20 column volume gradient of 0-300mM NaCl in tris pH 8.0. OPG protein was detected using a western blot of column fractions.

Fractions containing OPG were pooled and  
30 brought to a final concentration of 300mM NaCl, 0.2mM DTT. A NiNTA superose (Qiagen) column was equilibrated with 20mM tris pH 8.0, 300mM NaCl, 0.2mM DTT after which the pooled fractions were applied. The column was washed with equilibration buffer until baseline  
35 absorbance was reached. Proteins were eluted from the column with a 0-30mM Imidazole gradient in

equilibration buffer. Remaining proteins were washed off the column with 1M Imidazole. Again a western blot was used to detect OPG containing fractions.

Pooled fractions from the NiNTA column were  
5 dialyzed into 10mM potassium phosphate pH 7.0, 0.2mM DTT. The dialyzed pool was then applied to a ceramic hydroxyapatite column (Bio-Rad) which had been equilibrated in 10mM phosphate buffer. After column washing, the protein was eluted with a 10-100mM  
10 potassium phosphate gradient over 20 column volumes. This was then followed by a 20 column volume gradient of 100-400 mM phosphate.

OPG was detected by coomassie blue staining of SDS-polyacrylamide gels and by western blotting.  
15 Fractions were pooled and diafiltered onto PBS and frozen at -80°C. The purified protein runs as a monomer and will remain so after diafiltration into PBS. The monomer is stable when stored frozen or at pH 5 at 4°C. However if stored at 4°C in PBS, dimers and what  
20 appears to be trimers and tetramers will form after one week.

D. Purification of human OPG met[22-401] from E. coli

25 The bacterial cell paste was suspended into 10 mM EDTA to a concentration of 15% (w/v) using a low shear homogenizer at 5°C. The cells were then disrupted by two homogenizations at 15,000 psi each at 5°C. The resulting homogenate was centrifuged at 5,000  
30 x g for one hour at 5°C. The centrifugal pellet was washed by low shear homogenization into water at the original homogenization volume followed by centrifugation as before. The washed pellet was then solubilized to 15% (w/v) by a solution of (final  
35 concentration) 6 M guanidine HCl, 10 mM dithiothreitol, 10 mM TrisHCl, pH 8.5 at ambient temperature for 30



minutes. This solution was diluted 30-fold into 2M urea containing 50 mM CAPS, pH 10.5, 1 mM reduced glutathione and then stirred for 72 hours at 5°C. The OPG was purified from this solution at 25°C by first  
5 adjustment to pH 4.5 with acetic acid and then chromatography over a column of SP-HP Sepharose resin equilibrated with 25 mM sodium acetate, pH 4.5. The column elution was carried out with a linear sodium chloride gradient from 50 mM to 550 mM in the same  
10 buffer using 20 column volumes at a flow rate of 0.1 column volumes/minute. The peak fractions containing only the desired OPG form were pooled and stored at 5°C or buffer exchanged into phosphate buffered saline, concentrated by ultrafiltration, and then stored at  
15 5°C. This material was analyzed by reverse phase HPLC, SDS-PAGE, limulus amebocyte lysate assay for the presence of endotoxin, and N-terminal sequencing. In addition, techniques such as mass spectrometry, pH/temperature stability, fluorescence, circular  
20 dichroism, differential scanning calorimetry, and protease profiling assays may also be used to examine the folded nature of the protein.

25

#### EXAMPLE 11

##### Biological Activity of Recombinant OPG

Based on histology and histomorphometry, it appeared that hepatic overexpression of OPG in  
30 transgenic mice markedly decreased the numbers of osteoclasts leading to a marked increase in bone tissue (see Example 4). To gain further insight into potential mechanism(s) underlying this in vivo effect, various forms of recombinant OPG have been tested in an  
35 in vitro culture model of osteoclast formation (osteoclast forming assay). This culture system was

originally devised by Udagawa (Udagawa et al. Endocrinology 125, 1805-1813 (1989), Proc. Natl. Acad. Sci. USA 87, 7260-7264 (1990)) and employs a combination of bone marrow cells and cells from bone marrow stromal cell lines. A description of the modification of this culture system used for these studies has been previously published (Lacey et al. Endocrinology 136, 2367-2376 (1995)). In this method, bone marrow cells, flushed from the femurs and tibiae of mice, are cultured overnight in culture media (alpha MEM with 10% heat inactivated fetal bovine serum) supplemented with 500 U/ml CSF-1 (colony stimulating factor 1, also called M-CSF), a hematopoietic growth factor specific for cells of the monocyte/macrophage family lineage. Following this incubation, the non-adherent cells are collected, subjected to gradient purification, and then cocultured with cells from the bone marrow cell line ST2 ( $1 \times 10^6$  non-adherent cells :  $1 \times 10^5$  ST2 cells/ ml media). The media is supplemented with dexamethasone (100 nM) and the biologically-active metabolite of vitamin D3 known as 1,25 dihydroxyvitamin D3 ( $1,25 (OH)_2 D_3$ , 10 nM). To enhance osteoclast appearance, prostaglandin E2 (250 nM) is added to some cultures. The coculture period usually ranges from 8 - 10 days and the media, with all of the supplements freshly added, is renewed every 3-4 days. At various intervals, the cultures are assessed for the presence of tartrate acid phosphatase (TRAP) using either a histochemical stain (Sigma Kit # 387A, Sigma, St. Louis, MO) or TRAP solution assay. The TRAP histochemical method allows for the identification of osteoclasts phenotypically which are multinucleated (<sup>3</sup> 3 nuclei) cells that are also TRAP+. The solution assay involves lysing the osteoclast-containing cultures in a citrate buffer (100 mM, pH 5.0) containing 0.1% Triton X-100. Tartrate resistant acid

phosphatase activity is then measured based on the conversion of p-nitrophenylphosphate (20 nM) to p-nitrophenol in the presence of 80 mM sodium tartrate which occurs during a 3-5 minute incubation at RT. The  
5 reaction is terminated by the addition of NaOH to a final concentration of 0.5 M. The optical density at 405 nm is measured and the results are plotted.

Previous studies (Udagawa et al. ibid) using the osteoclast forming assay have demonstrated that  
10 these cells express receptors for  $^{125}\text{I}$ -calcitonin (autoradiography) and can make pits on bone surfaces, which when combined with TRAP positivity confirm that the multinucleated cells have an osteoclast phenotype. Additional evidence in support of the osteoclast  
15 phenotype of the multinucleated cells that arise in vitro in the osteoclast forming assay are that the cells express  $\alpha\nu$  and  $\beta 3$  integrins by immunocytochemistry and calcitonin receptor and TRAP mRNA by in situ hybridization (ISH).

20 The huOPG [22-401]-Fc fusion was purified from CHO cell conditioned media and subsequently utilized in the osteoclast forming assay. At 100 ng/ml of huOPG [22-401]-Fc, osteoclast formation was virtually 100% inhibited (Figure 19A). The levels of  
25 TRAP measured in lysed cultures in microtitre plate wells were also inhibited in the presence of OPG with an  $\text{ID}_{50}$  of approximately 3 ng/ml (Figure 20). The level of TRAP activity in lysates appeared to correlate with the relative number of osteoclasts seen by TRAP  
30 cytochemistry (compare Figures 19A-19G and 20). Purified human IgG1 and TNFbp were also tested in this model and were found to have no inhibitory or stimulatory effects suggesting that the inhibitory effects of the huOPG [22-401]-Fc were due to the OPG  
35 portion of the fusion protein. Additional forms of the

human and murine molecules have been tested and the cumulative data are summarized in Table 1.

5

Table 1  
Effects of various OPG forms on in vitro  
osteoclast formation

10	<u>OPG Construct</u>	<u>Relative Bioactivity in vitro</u>
	muOPG [22-401]-Fc	+++
	muOPG [22-194]-Fc	+++
	muOPG [22-185]-Fc	++
15	muOPG [22-180]-Fc	-
	muOPG [22-401]	+++
	muOPG [22-401] C195	+++
	muOPG [22-401] C202	+
	muOPG [22-401] C277	-
20	muOPG [22-401] C319	+
	muOPG [22-401] C400	+
	muOPG [22-185]	-
	muOPG [22-194]	++
	muOPG [22-200]	++
25	muOPG [22-212]	-
	muOPG [22-293]	+++
	muOPG [22-355]	+++
	huOPG [22-401]-Fc	+++
30	huOPG [22-201]-Fc	+++
	huOPG [22-401]-Fc P26A	+++
	huOPG [22-401]-Fc Y28F	+++
	huOPG [22-401]	+++
	huOPG [27-401]-Fc	++
35	huOPG [29-401]-Fc	++
	huOPG [32-401]-Fc	+/-

- +++ , ED<sub>50</sub> = 0.4-2 ng/ml  
++ , ED<sub>50</sub> = 2-10 ng/ml  
+ , ED<sub>50</sub> = 10-100 ng/ml  
5 - , ED<sub>50</sub> > 100 ng/ml

The cumulative data suggest that murine and human OPG amino acid sequences 22-401 are fully active in vitro, when either fused to the Fc domain, or  
10 unfused. They inhibit in a dose-dependent manner and possess half-maximal activities in the 2-10 ng/ml range. Truncation of the murine C-terminus at threonine residue 180 inactivates the molecule, whereas truncations at cysteine 185 and beyond have full  
15 activity. The cysteine residue located at position 185 is predicted to form an SS3 bond in the domain 4 region of OPG. Removal of this residue in other TNFR-related proteins has previously been shown to abrogate biological activity (Yan et al. J. Biol. Chem. 266,  
20 12099-12104 (1994)). Our finding that muOPG[22-180]-Fc is inactive while muOPG[22-185]-Fc is active is consistent with these findings. This suggests that amino acid residues 22-185 define a region for OPG activity.

25 These findings indicate that like transgenically-expressed OPG, recombinant OPG protein also suppressed osteoclast formation as tested in the osteoclast forming assay. Time course experiments examining the appearance of TRAP+ cells,  $\beta$ 3+ cells,  
30 F480+ cells in cultures continuously exposed to OPG demonstrate that OPG blocks the appearance TRAP+ and  $\beta$ 3+ cells, but not F480+ cells. In contrast, TRAP+ and  $\beta$ 3+ cells begin to appear as early as day 4 following culture establishment in control cultures. Only F480+  
35 cells can be found in OPG-treated cultures and they

appear to be present at qualitatively the same numbers as the control cultures. Thus, the mechanism of OPG effects in vitro appears to involve a blockade in osteoclast differentiation at a step beyond the  
5 appearance of monocyte-macrophages but before the appearance of cells expressing either TRAP or  $\beta 3$  integrins. Collectively these findings indicate that OPG does not interfere with the general growth and differentiation of monocyte-macrophage precursors from  
10 bone marrow, but rather suggests that OPG specifically blocks the selective differentiation of osteoclasts from monocyte-macrophage precursors.

To determine more specifically when in the osteoclast differentiation pathway that OPG was  
15 inhibitory, a variation of the in vitro culture method was employed. This variation, described in (Lacey et al. supra), employs bone marrow macrophages as osteoclast precursors. The osteoclast precursors are derived by taking the nonadherent bone marrow cells  
20 after an overnight incubation in CSF-1/M-CSF, and culturing the cells for an additional 4 days with 1,000 - 2,000 U/ml CSF-1. Following 4 days of culture, termed the growth phase, the non-adherent cells are removed. The adherent cells, which are bone marrow  
25 macrophages, can then be exposed for up to 2 days to various treatments in the presence of 1,000 - 2,000 U/ml CSF-1. This 2 day period is called the intermediate differentiation period. Thereafter, the cell layers are again rinsed and then ST-2 cells ( $1 \times 10^5$  cell/ml), dexamethasone (100 nM) and 1,25 (OH) $_2$  D $_3$   
30 (10 nM) are added for the last 8 days for what is termed the terminal differentiation period. Test agents can be added during this terminal period as well. Acquisition of phenotypic markers of osteoclast  
35 differentiation are acquired during this terminal period (Lacey et al. ibid).

huOPG [22-401]-Fc (100 ng/ml) was tested for its effects on osteoclast formation in this model by adding it during either the intermediate, terminal or, alternatively, both differentiation periods. Both TRAP  
5 cytochemistry and solution assays were performed. The results of the solution assay are shown in Figure 21. HuOPG [22-401]-Fc inhibited the appearance of TRAP activity when added to both the intermediate and terminal or only the terminal differentiation phases.  
10 When added to the intermediate phase and then removed from the cultures by rinsing, huOPG [22-401]-Fc did not block the appearance of TRAP activity in culture lysates. The cytochemistry results parallel the solution assay data. Collectively, these observations  
15 indicate that huOPG [22-401]-Fc only needs to be present during the terminal differentiation period for it to exert its all of its suppressive effects on osteoclast formation.

B. In vivo IL1- $\alpha$  and IL1- $\beta$  challenge experiments

20 IL1 increases bone resorption both systemically and locally when injected subcutaneously over the calvaria of mice (Boyce et al., Endocrinology 125, 1142-1150 (1989)). The systemic effects can be assessed by the degree of hypercalcemia and the local  
25 effects histologically by assessing the relative magnitude of the osteoclast-mediated response. The aim of these experiments was to determine if recombinant muOPG [22-401]-Fc could modify the local and/or systemic actions of IL1 when injected subcutaneously  
30 over the same region of the calvaria as IL1.

IL-1  $\beta$  experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per  
35 group): Control group: IL1 treated animals (mice received 1 injection/day of 2.5 ug of IL1- $\beta$ ); Low dose

muOPG [22-401]-Fc treated animals (mice received 3 injections/day of 1  $\mu$ g of muOPG [22-401]-Fc); Low dose muopg [22-401]-Fc and IL1- $\beta$ ; High dose muOPG [22-401]-Fc treated animals (mice receive 3 injections/day of 10  $\mu$ g muOPG [22-401]-Fc); High dose muOPG [22-401]-Fc and IL1- $\beta$ . All mice received the same total number of injections of either active factor or vehicle (0.1% bovine serum albumin in phosphate buffered saline). All groups are sacrificed on the day after the last injection. The weights and blood ionized calcium levels are measured before the first injections, four hours after the second injection and 24 hours after the third IL1 injection, just before the animals were sacrificed. After sacrifice the calvaria were removed and processed for paraffin sectioning.

#### IL1- $\alpha$ experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group; IL1 alpha treated animals (mice received 1 injection/day of 5  $\mu$ g of IL1-alpha); Low dose muOPG [22-401]-Fc treated animals (mice received 1 injection/day of 10  $\mu$ g of muOPG [22-401]-Fc; Low dose muopg [22-401]-Fc and IL1-alpha, (dosing as above); High dose muopg [22-401]-Fc treated animals (mice received 3 injections/day of 10  $\mu$ g muOPG [22-401]-Fc; High dose muOPG [22-401]-Fc and IL1- $\alpha$ . All mice received the same number of injections/day of either active factor or vehicle. All groups were sacrificed on the day after the last injection. The blood ionized calcium levels were measured before the first injection, four hours after the second injection and 24 hours after the third IL1 injection, just before the animals were sacrificed. The animal weights were measured before the first injection, four hours after the second injection and 24 hours after the third IL1



injection, just before the animals were sacrificed. After sacrifice the calvaria were removed and processed for paraffin sectioning.

5

#### Histological methods

Calvarial bone samples were fixed in zinc formalin, decalcified in formic acid, dehydrated through ethanol and mounted in paraffin. Sections (5µm  
10 thick) were cut through the calvaria adjacent to the lambdoid suture and stained with either hematoxylin and eosin or reacted for tartrate resistant acid phosphatase activity (Sigma Kit# 387A) and counterstained with hematoxylin. Bone resorption was  
15 assessed in the IL1-α treated mice by histomorphometric methods using the Osteomeasure (Osteometrics, Atlanta, GA) by tracing histologic features onto a digitizer platen using a microscope-mounted camera lucida attachment. Osteoclast numbers, osteoclast lined  
20 surfaces, and eroded surfaces were determined in the marrow spaces of the calvarial bone. The injected and non-injected sides of the calvaria were measured separately.

#### 25 Results

IL1-α and IL1-β produced hypercalcemia at the doses used, particularly on the second day, presumably by the induction of increased bone resorption systemically. The hypercalcemic response was blocked  
30 by muOPG [22-401]-Fc in the IL1-beta treated mice and significantly diminished in mice treated with IL1-alpha, an effect most apparent on day 2 (Figure 22A-22B).

Histologic analysis of the calvariae of mice  
35 treated with IL1-alpha and beta shows that IL1 treatments alone produce a marked increase in the

indices of bone resorption including: osteoclast number, osteoclast lined surface, and eroded surface (surfaces showing deep scalloping due to osteoclastic action (Figure 23B, Table 2). In response to IL1- $\alpha$  or IL1- $\beta$ , the increases in bone resorption were similar on the injected and non-injected sides of the calvaria. Muopg [22-401]-Fc injections reduced bone resorption in both IL1-alpha and beta treated mice and in mice receiving vehicle alone but this reduction was seen only on the muopg [22-401]-Fc injected sides of the calvariae.

The most likely explanation for these observations is that muOPG [22-401]-Fc inhibited bone resorption, a conclusion supported by the reduction of both the total osteoclast number and the percentage of available bone surface undergoing bone resorption, in the region of the calvaria adjacent to the muOPG [22-401]-Fc injection sites. The actions of muOPG [22-401]-Fc appeared to be most marked locally by histology, but the fact that muOPG [22-401]-Fc also blunted IL1-induced hypercalcemia suggests that muOPG [22-401]-Fc has more subtle effects on bone resorption systemically.

Table 2. Effects of OPG on variables of bone resorption in IL-1 injected mice.

	Osteoclast Surface % Bone Surface (mean $\pm$ S.D)		Eroded Surface %Bone Surface (mean $\pm$ S.D)		Osteoclast Number/mm <sup>2</sup> Tissue Area (mean $\pm$ S.D)	
	Non-injected side	Injected side	Non-injected side	Injected side	Non-injected side	Injected side
<b>Experiment 1</b>						
Control	12.36 $\pm$ 3.44	9.54 $\pm$ 2.46	8.07 $\pm$ 3.90	9.75 $\pm$ 3.16	32.51 $\pm$ 11.09	23.50 $\pm$ 10.83
IL-1 $\beta$ (2.5 $\mu$ g/d)	17.18 $\pm$ 1.30	16.40 $\pm$ 2.16	40.66 $\pm$ 4.28	37.53 $\pm$ 10.28	71.80 $\pm$ 18.76	60.89 $\pm$ 5.16
OPG (40 $\mu$ g/d)	10.12 $\pm$ 3.71	5.04 $\pm$ 1.66	9.73 $\pm$ 4.33	4.19 $\pm$ 3.61	32.73 $\pm$ 11.09	15.24 $\pm$ 7.54
OPG+IL-1 $\beta$	18.61 $\pm$ 2.46	# 13.26 $\pm$ 2.50	44.87 $\pm$ 8.63	# 25.94 $\pm$ 6.82	69.42 $\pm$ 36.29	# 47.13 $\pm$ 24.26
<b>Experiment 2</b>						
Control	11.56 $\pm$ 4.22	11.95 $\pm$ 2.97	12.67 $\pm$ 5.04	10.03 $\pm$ 5.13	51.72 $\pm$ 23.93	56.03 $\pm$ 30.70
IL-1 $\alpha$ (5 $\mu$ g/d)	28.81 $\pm$ 4.84	23.46 $\pm$ 5.76	37.51 $\pm$ 5.16	41.10 $\pm$ 12.53	113.60 $\pm$ 18.04	102.70 $\pm$ 32.09
OPG (40 $\mu$ g/d)	14.40 $\pm$ 1.00	# 4.26 $\pm$ 2.54	11.55 $\pm$ 4.14	# 4.29 $\pm$ 3.16	72.28 $\pm$ 14.11	# 22.65 $\pm$ 16.68
OPG+IL-1 $\alpha$	29.58 $\pm$ 8.80	# 17.83 $\pm$ 3.34	33.66 $\pm$ 9.21	# 24.38 $\pm$ 8.88	146.10 $\pm$ 42.37	# 66.56 $\pm$ 15.62

# Different to non-injected side p < 0.05 (by paired t test)

C. Systemic Effects of muOPG [22-401]-Fc in Growing Mice

Male BDF1 mice aged 3-4 weeks, weight range 9.2- 15.7g were divided into groups of ten mice per group. These mice were injected subcutaneously with saline or muOPG [22-401]-Fc 2.5mg/kg *bid* for 14 days (5mg/kg/day). The mice were radiographed before treatment, at day 7 and on day 14. The mice were sacrificed 24 hours after the final injection. The right femur was removed, fixed in zinc formalin, decalcified in formic acid and embedded in paraffin. Sections were cut through the mid region of the distal femoral metaphysis and the femoral shaft. Bone density, by histomorphometry, was determined in six adjacent regions extending from the metaphyseal limit of the growth plate, through the primary and secondary spongiosa and into the femoral diaphysis (shaft). Each region was 0.5 X 0.5 mm<sup>2</sup>.

Radiographic changes

After seven days of treatment there was evidence of a zone of increased bone density in the spongiosa associated with the growth plates in the OPG treated mice relative to that seen in the controls. The effects were particularly striking in the distal femoral and the proximal tibial metaphases (Figure 24A-24B). However bands of increased density were also apparent in the vertebral bodies, the iliac crest and the distal tibia. At 14 days, the regions of opacity had extended further into the femoral and tibial shafts though the intensity of the radio-opacity was diminished. Additionally, there were no differences in the length of the femurs at the completion of the experiment or in the change in length over the duration

of the experiment implying that OPG does not alter bone growth.

#### Histological Changes

5           The distal femoral metaphysis showed  
increased bone density in a regions 1.1 to 2.65 mm in  
distance from the growth plate (Figures 25 and 26A-  
26B). This is a region where bone is rapidly removed  
by osteoclast-mediated bone resorption in mice. In  
10 these rapidly growing young mice, the increase in bone  
in this region observed with OPG treatment is  
consistent with an inhibition of bone resorption.

#### D. Effects of Osteoprotegerin on Bone Loss Induced by 15 Ovariectomy in the Rat

Twelve week old female Fisher rats were  
ovariectomized (OVX) or sham operated and dual xray  
absorptiometry (DEXA) measurements made of the bone  
20 density in the distal femoral metaphysis. After 3  
days recovery period, the animals received daily  
injections for 14 days as follows: Ten sham operated  
animals received vehicle (phosphate buffered saline);  
Ten OVX animals received vehicle (phosphate buffered  
25 saline); Six OVX animals received OPG-Fc 5mg/kg SC;  
Six OVX animals received pamidronate (PAM) 5mg/kg SC;  
Six OVX animals received estrogen (ESTR) 40ug/kg SC.  
After 7 and 14 days treatment the animals had bone  
density measured by DEXA. Two days after the last  
30 injection the animals were killed and the right tibia  
and femur removed for histological evaluation.

The DEXA measurements of bone density showed a  
trend to reduction in the bone density following  
35 ovariectomy that was blocked by OPG-Fc. Its effects  
were similar to the known antiresorptive agents  
estrogen and pamidronate. (Figure 27). The

histomorphometric analysis confirmed these observations with OPG-Fc treatment producing a bone density that was significantly higher in OVX rats than that seen in untreated OVX rats (Figure 28). These results confirm the activity of OPG in the bone loss associated with withdrawal of endogenous estrogen following ovariectomy.

10 In vivo Summary

The in vivo actions of recombinant OPG parallel the changes seen in OPG transgenic mice. The reduction in osteoclast number seen in the OPG transgenic is reproduced by injecting recombinant OPG locally over the calvaria in both normal mice and in mice treated with IL1- $\alpha$  or IL1- $\beta$ . The OPG transgenic mice develop an osteopetrotic phenotype with progressive filling of the marrow cavity with bone and unremodelled cartilage extending from the growth plates from day 1 onward after birth. In normal three week old (growing) mice, OPG treatments also led to retention of bone and unremodelled cartilage in regions of endochondral bone formation, an effect observed radiographically and confirmed histologically. Thus, recombinant OPG produces phenotypic changes in normal animals similar to those seen in the transgenic animals and the changes are consistent with OPG-induced inhibition of bone resorption. Based on in vitro assays of osteoclast formation, a significant portion of this inhibition is due to impaired osteoclast formation. Consistent with this hypothesis, OPG blocks ovariectomy-induced osteoporosis in rat. Bone loss in this model is known to be mediated by activated osteoclasts, suggesting a role for OPG in treatment of primary osteoporosis.

EXAMPLE 12

Pegylation Derivatives of OPG

5

Preparation of N-terminal PEG-OPG conjugates by reductive alkylation

HuOPG met [22-194] P25A was buffer exchanged into 25-50 mM NaOAc, pH 4.5-4.8 and concentrated to 2-5 mg/ml. This solution was used to conduct OPG reductive alkylation with monofunctional PEG aldehydes at 5-7 °C. PEG monofunctional aldehydes, linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 2-4 moles of PEG aldehyde per mole of OPG. After dissolution of polymer into the protein solution, sodium cyanoborohydride was added to give a final concentration of 15 to 20 mM in the reaction mixture from 1-1.6 M freshly prepared stock solution in cold DI water. The progress of the reaction and the extent of OPG PEGylation was monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO<sub>4</sub>, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 16-18 hours, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were pooled and characterized by SEC HPLC and SDS-PAGE. By N-terminal sequencing, it was determined that the monoPEG-OPG conjugate, the major reaction product in most cases, was 98% N-terminally PEG-modified OPG.

This procedure was generally used to prepare the following N-terminal PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A: 5 kD monoPEG, 10 kD mono branched PEG, 12 kD monoPEG, 20 kD monoPEG, 20 kD mono branched PEG, 25 kD monoPEG, 31 kD monoPEG, 57 kD monoPEG, 12 kD diPEG, 25 kD diPEG, 31 kD diPEG, 57 kD diPEG, 25 kD triPEG.

Preparation of PEG-OPG conjugates by acylation

10 HuOPG met [22-194] P25A was buffer exchanged into 50 mM BICINE buffer, pH 8 and concentrated to 2-3 mg/ml. This solution was used to conduct OPG acylation with monofunctional PEG N-hydroxysuccinimidyl esters at room temperature. PEG N-hydroxysuccinimidyl esters,  
15 linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 4-8 moles of PEG N-hydroxysuccinimidyl ester per mole of OPG. The progress of the reaction and the extent of OPG PEGylation was  
20 monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO<sub>4</sub>, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 1 hour, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The  
25 reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were  
30 pooled and characterized by SEC HPLC and SDS-PAGE.

This procedure was generally used to prepare the following PEG-OPG conjugates: 5 kD polyPEG, 20 kD polyPEG, 40 kD poly branched PEG, 50 kD poly PEG.



Preparation of dimeric PEG-OPG

HuOPG met [22-194] P25A is prepared for  
thiolation at 1-3 mg/ml in a phosphate buffer at near  
neutral pH. S-acetyl mecaptosuccinic anhydride (AMSA)  
5 is added in a 3-7 fold molar excess while maintaining  
pH at 7.0 and the rxn stirred at 4\_C for 2 hrs. The  
monothiolated-OPG is separated from unmodified and  
polythiolated OPG by ion exchange chromatography and  
the protected thiol deprotected by treatment with  
10 hydroxylamine. After deprotection, the hydroxylamine  
is removed by gel filtration and the resultant  
monothiolated-OPG is subjected to a variety of thiol  
specific crosslinking chemistries. To generate a  
disulfide bonded dimer, the thiolated OPG at >1mg/ml is  
15 allowed to undergo air oxidation by dialysis in  
slightly basic phosphate buffer. The covalent  
thioether OPG dimer was prepared by reacting the bis-  
maleimide crosslinker, N,N-bis(3-maleimido propianyl)-  
2-hydroxy 1,3 propane with the thiolated OPG at >1mg/ml  
20 at a 0.6x molar ratio of crosslinker:OPG in phosphate  
buffer at pH 6.5. Similarly, the PEG dumbbells are  
produced by reaction of substoichiometric amounts of  
bis-maleimide PEG crosslinkers with thiolated OPG at  
>1mg/ml in phosphate buffer at pH 6.5. Any of the  
25 above dimeric conjugates may be further purified using  
either ion exchange or size exclusion chromatographies.

Dimeric PEG-OPG conjugates (where OPG is HuOPG met  
[22-194] P25A prepared using the above procedures  
include disulfide-bonded OPG dimer, covalent thioether  
30 OPG dimer with an aliphatic amine type crosslinker, 3.4  
kD and 8kD PEG dumbbells and monobells.

PEG-OPG conjugates were tested for activity in  
vitro using the osteoclast maturation assay described  
in Example 11A and for activity in vivo by measuring  
35 increased bone density after injection into mice as

described in Example 11C. The in vivo activity is shown below in Table 3.

Table 3

5           In vivo biological activity of Pegylated OPG

	<u>OPG Construct</u>	<u>Increase in Tibial Bone Density</u>
	muOPG met [22-194]	-
10	muOPG met [22-194] 5k PEG	+
	muOPG met [22-194] 20k PEG	+
	huOPG met [22-194] P25A	-
	huOPG met [22-194] P25A 5k PEG	+
15	huOPG met [22-194] P25A 20k PEG	+
	huOPG met [22-194] P25A 31k PEG	+
	huOPG met [22-194] P25A 57k PEG	+
	huOPG met [22-194] P25A 12k PEG	+
	huOPG met [22-194] P25A 20k Branched PEG	+
20	huOPG met [22-194] P25A 8k PEG dimer	+
	huOPG met [22-194] P25A disulfide crosslink	+

\* \* \*

While the invention has been described in  
what is considered to be its preferred embodiments, it  
5 is not to be limited to the disclosed embodiments, but  
on the contrary, is intended to cover various  
modifications and equivalents included within the  
spirit and scope of the appended claims, which scope is  
to be accorded the broadest interpretation so as to  
10 encompass all such modifications and equivalents.

20250303 14:00:00

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Amgen Inc.

(ii) TITLE OF INVENTION: OSTEOPROTEGERIN

(iii) NUMBER OF SEQUENCES: 168

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Amgen Inc.
- (B) STREET: 1840 Dehavilland Drive
- (C) CITY: Thousand Oaks
- (D) STATE: California
- (E) COUNTRY: United States
- (F) ZIP: 91320

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Winter, Robert B.
- (C) REFERENCE/DOCKET NUMBER: A-378-CIP2

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAAGGAAGGA AAAAAGCGGC CGCTACANNN NNNNNT

36

5 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGACCCACG CGTCCG

16

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGTGCGCAG GC

12

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTAAAACGA CGGCCAGT

18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGGAAACAG CTATGACC

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAATTAACCC TCACTAAAGG

20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCATTATGAC CCAGAAACCG GAC

23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

10

AGGTAGCGCC CTTCTCACA TTC

23

(2) INFORMATION FOR SEQ ID NO:9:

15

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

25

GACTAGTCCC ACAATGAACA AGTGGCTGTG

30

(2) INFORMATION FOR SEQ ID NO:10:

30

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 45 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

40

ATAAGAATGC GGCCGCTAAA CTATGAAACA GCCCAGTGAC CATTC

45

(2) INFORMATION FOR SEQ ID NO:11:

45

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCTCTAGAA AGAGCTGGGA C

21

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCCGTGTTC CATTTATGAG C

21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATCAAAGGCA GGGCATACTT CCTG

24

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTTGCACTCC TGTTTCACGG TCTG

24

(2) INFORMATION FOR SEQ ID NO:15:



- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 24 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAAGACACCT TGAAGGCCT GATG

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 24 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAACTTTTAC AGAAGAGCAT CAGC

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 33 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCGCGGCCG CATGAACAAG TGGCTGTGCT GCG

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 31 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTCTAGAG AAACAGCCCA GTGACCATTC C

31

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTGAAGCTGT GCAAGAACCT GATG

24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATCAAAGGCA GGGCATACTT CCTG

24

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

5 CAGATCCTGA AGCTGCTCAG TTTG

24

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

20 AGCGCGGCCG CGGGGACCAC AATGAACAAG TTG

33

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

35 AGCTCTAGAA TTGTGAGGAA ACAGCTCAAT GGC

33

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATAGCGGCCG CTGAGCCCAA ATCTTGTGAC AAAACTCAC

39

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCTAGAGTCG ACTTATCATT TACCCGGAGA CAGGGAGAGG CTCTT

45

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCTCTGAGCT CAAGCTTCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCTCTGCGGC CGCTAAGCAG CTTATTTTCA CGGATTGAAC CTG

43

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 38 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 24 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCCGTAAGAA ACAGCCCAGT GACC

24

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 31 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CCTCTGCGGC CGCTGTTGCA TTCCTTTCT G

31

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: protein

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His  
1 5 10 15

15

Gln Leu Leu

(2) INFORMATION FOR SEQ ID NO:32:

20

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCCCTTGCCC TGACCACTCT T

21

35

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

50

CCTCTGCGGC CGCACACACG TTGTCATGTG TTGC

34

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 21 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 34 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCTCTGCGGC CGCCTTTTGC GTGGCTTCTC TGTT

34

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 37 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG

37

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCTCTGCGGC CGCTAAGCAG CTTATTTTCTA CTGAATGG

38

(2) INFORMATION FOR SEQ ID NO:38:

15

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCTCTGAGCT CAAGCTTGGT TTCCGGGGAC CACAATG

37

30

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

45

CCTCTGCGGC CGCCAGGGTA ACATCTATTC CAC

33

(2) INFORMATION FOR SEQ ID NO:40:

50

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 35 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single



(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CCGAAGCTTC CACCATGAAC AAGTGGCTGT GCTGC

10

35

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CCTCTGTCGA CTATTATAAG CAGCTTATTT TCACGGATTG

25

40

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TCCCTTGCCC TGACCACTCT T

40

21

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCTCTGTCGA CTTAACACAC GTTGTCATGT GTTGC

35

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TCCCTTGCCC TGACCACTCT T

21

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCTCTGTCGA CTTACTTTTG CGTGGCTTCT CTGTT

35

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1537 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTGAAGAGCG TGAAGAGCGG TTCCTCCTTT CAGCAAAAAA CCCCTCAAGA CCCGTTTAGA 60  
5 GGCCCAAGG GGTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAAC T CAGCTTCCTT 120  
TCGGGCTTTC TTCTTCTTCT TCTTCTTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC 180  
10 CTGCAGGTCG AACTAGTGA GCTCGAATTC CAACGCGTTA ACCATATGTT ATTCCTCCTT 240  
TAATTAGTTA AAACAAATCT AGAATCAAAT CGATTAATCG ACTATAACAA ACCATTTTCT 300  
TGCGTAAACC TGTACGATCC TACAGGTAAT TATGTTAAAC AATTGTATTT CAAGCGATAT 360  
15 AATAGTGTGA CAAAAATCCA ATTTATTAGA ATCAAATGTC AATCTATTAC CGTTTTAATG 420  
ATATATAACA CGCAAAACTT GCGACAAACA ATAGGTAAGG ATAAAGAGAT GGGTATGAAA 480  
GACATAAATG CCGACGACAC TTACAGAATA ATTAATAAAA TTAAAGCCTG TAGAAGCAAT 540  
20 AATGATATTA ATCAATGCTT ATCTGATATG ACTAAAATGG TACATTGTGA ATATTATTTA 600  
CTCGCGATCA TTTATCCTCA TTCTATGGTT AAATCTGATA TTTCAATTCT GGATAATTAC 660  
25 CCTAAAAAAT GGAGGCAATA TTATGATGAC GCTAATTTAA TAAAATATGA TCCTATAGTA 720  
GATTATTCTA ACTCCAATCA TTCACCGATT AATTGGAATA TATTTGAAAA CAATGCTGTA 780  
AATAAAAAAT CTCCAAATGT AATTAAAGAA GCGAAATCAT CAGGTCTTAT CACTGGGTTT 840  
30 AGTTTCCCTA TTCATACTGC TAATAATGGC TTCGGAATGC TTAGTTTTGC ACATTCAGAG 900  
AAAGACAACT ATATAGATAG TTTATTTTTA CATGCGTGTA TGAACATACC ATTAATTGTT 960  
35 CCTTCTCTAG TTGATAATTA TCGAAAAATA AATATAGCAA ATAATAAATC AAACAACGAT 1020  
TTAACCAAAA GAGAAAAAGA ATGTTTAGCG TGGGCATGCG AAGGAAAAAG CTCTTGGGAT 1080  
ATTTCAAAAA TATTAGGCTG TAGTAAGCGC ACGGTCACCT TCCATTTAAC CAATGCGCAA 1140  
40 ATGAAACTCA ATACAACAAA CCGCTGCCAA AGTATTTCTA AAGCAATTTT AACAGGAGCA 1200  
ATTGATTGCC CATACTTTAA AAGTTAAGTA CGACGTCCAT ATTTGAATGT ATTTAGAAAA 1260  
45 ATAAACAAAA GAGTTTGTAG AAACGCAAAA AGGCCATCCG TCAGGATGGC CTTCTGCTTA 1320  
ATTTGATGCC TGGCAGTTTA TGGCGGGCGT CCTGCCCCG ACCCTCCGGG CCGTTGCTTC 1380  
GCAACGTTCA AATCCGCTCC CGGCGGATTT GTCCTACTCA GGAGAGCGTT CACCGACAAA 1440  
50 CAACAGATAA AACGAAAGGC CCAGTCTTTC GACTGAGCCT TTCGTTTTAT TTGATGCCTG 1500  
GCAGTTCCTT ACTCTCGCAT GGGGAGACCA TGCATAC 1537

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGAATTC GGTAC

55

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CGAATTCCAA CGCGTTAACC ATATGTTATT CCTCCTTCTA GAATCAAAT

49

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1546 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GCGTAACGTA TGCATGGTCT CCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA 60  
CGAAAGGCTC AGTCGAAAGA CTGGGCCTTT CGTTTTATCT GTTGTGTGTC GGTGAACGCT 120  
CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTTGAACG TTGCGAAGCA ACGGCCCCGA 180  
GGGTGGCGGG CAGGACGCCC GCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGCCATC 240  
CTGACGGATG GCCTTTTTCG GTTCTACAA ACTCTTTTGT TTATTTTCT AAATACATTC 300  
AAATATGGAC GTCGTACTTA ACTTTTAAAG TATGGGCAAT CAATTGCTCC TGTAAAAAT 360  
GCTTTAGAAA TACTTTGGCA GCGGTTTGTT GTATTGAGTT TCATTTGCGC ATTGGTTAAA 420  
TGGAAAGTGA CCGTGCGCTT ACTACAGCCT AATATTTTGG AAATATCCCA AGAGCTTTTT 480  
CCTTCGCATG CCCACGCTAA ACATTCTTTT TCTCTTTTGG TTAAATCGTT GTTTGATTTA 540  
TTATTTGCTA TATTTATTTT TCGATAATTA TCAACTAGAG AAGGAACAAT TAATGGTATG 600  
TTCATACACG CATGTAAAAA TAAACTATCT ATATAGTTGT CTTTCTCTGA ATGTGCAAAA 660  
CTAAGCATTC CGAAGCCATT ATTAGCAGTA TGAATAGGGA AACTAAACCC AGTGATAAGA 720  
CCTGATGATT TCGCTTCTTT AATTACATTT GGAGATTTTT TATTTACAGC ATTGTTTTCA 780  
AATATATTCC AATTAATCGG TGAATGATTG GAGTTAGAAT AATCTACTAT AGGATCATAT 840  
TTTATTAAAT TAGCGTCATC ATAATATTGC CTCCATTTTT TAGGGTAATT ATCCAGAATT 900  
GAAATATCAG ATTTAACCAT AGAATGAGGA TAAATGATCG CGAGTAAATA ATATTCACAA 960  
TGTACCATTT TAGTCATATC AGATAAGCAT TGATTAATAT CATTATTGCT TCTACAGGCT 1020  
TTAATTTTAT TAATTATTCT GTAAGTGTG TCGGCATTTA TGTCTTTCAT ACCCATCTCT 1080  
TTATCCTTAC CTATTGTTTG TCGCAAGTTT TCGTGTTAT ATATCATTA AACGGTAATA 1140

GATTGACATT TGATTCTAAT AAATTGGATT TTTGTCACAC TATTATATCG CTTGAAATAC 1200  
 AATTGTTTAA CATAAGTACC TGTAGGATCG TACAGGTTTA CGCAAGAAAA TGGTTTGTTA 1260  
 5 TAGTCGATTA ATCGATTTGA TTCTAGATTT GTTTTAACTA ATTAAAGGAG GAATAACATA 1320  
 TGGTTAACGC GTTGGAATTC GAGCTCACTA GTGTCGACCT GCAGGGTACC ATGGAAGCTT 1380  
 10 ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC CCGAAAGGAA GCTGAGTTGG 1440  
 CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG GGCCTCTAAA CGGGTCTTGA 1500  
 GGGGTTTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT TCACGC 1546

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 47 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TATGAAACAT CATCACCATC ACCATCATGC TAGCGTTAAC GCGTTGG 47

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 49 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

AATTCCAACG CGTTAACGCT AGCATGATGG TGATGGTGAT GATGTTTCA 49

(2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 141 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CTAATTCCGC TCTCACCTAC CAAACAATGC CCCCCTGCAA AAAATAAAATT CATATAAAAA 60  
15 ACATACAGAT AACCATCTGC GGTGATAAAT TATCTCTGGC GGTGTTGACA TAAATACCAC 120  
TGGCGGTGAT ACTGAGCACA T 141

20

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 147 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CGATGTGCTC AGTATCACCG CCAGTGGTAT TTATGTCAAC ACCGCCAGAG ATAATTTATC 60  
35 ACCGCAGATG GTTATCTGTA TGTTTTTTAT ATGAATTTAT TTTTTCAGG GGGGCATTGT 120  
TTGGTAGGTG AGAGCGGAAT TAGACGT 147

40

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 55 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGAATTC GGTAC

55

5

(2) INFORMATION FOR SEQ ID NO:56:

10

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 49 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

15

- (ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CGAATTCCAA CGCGTTAACC ATATGTTATT CCTCCTTCTA GAATCAAAT

49

25

(2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 668 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

30

- (ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

40	GTGAAGAGCG TGAAGAGCGG TTCCTCCTTT CAGCAAAAAA CCCCTCAAGA CCCGTTTAGA	60
	GGCCCCAAGG GGTATGCTA GTTATTGCTC AGCGGTGGCA GCAGCCAACT CAGCTTCCTT	120
	TCGGGCTTTC TTCTTCTTCT TCTTCTTTCC GCGGATCCTC GAGTAAGCTT CCATGGTACC	180
45	CTGCAGGTCG AACTAGTGA GCTCGAATTC CAACGCGTTA ACCATATGTT ATTCCTCCTT	240
	TAATTAGTTA ACTCAAATCT AGAATCAAAT CGATAAATTG TGAGCGCTCA CAATTGAGAA	300
50	TATTAATCAA GAATTTTAGC ATTTGTCAAA TGAATTTTTT AAAAATTATG AGACGTCCAT	360
	ATTTGAATGT ATTTAGAAAA ATAAACAAAA GAGTTTGTAG AAACGCAAAA AGGCCATCCG	420



TCAGGATGGC CTTCTGCTTA ATTTGATGCC TGGCAGTTTA TGGCGGGCGT CCTGCCCCGCC 480  
ACCCCTCCGGG CCGTTGCTTC GCAACGTTCA AATCCGCTCC CGGCGGATTT GTCCTACTCA 540  
5 GGAGAGCGTT CACCGACAAA CAACAGATAA AACGAAAGGC CCAGTCTTTC GACTGAGCCT 600  
TTCGTTTTAT TTGATGCCTG GCAGTTCCTT ACTCTCGCAT GGGGAGACCA TGCATACGTT 660  
ACGCACGT 668

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 726 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GCGTAACGTA TGCATGGTCT CCCCATGCGA GAGTAGGGAA CTGCCAGGCA TCAAATAAAA 60  
CGAAAGGCTC AGTCGAAAGA CTGGGCCTTT CGTTTTATCT GTTGTTTGTC GGTGAACGCT 120  
30 CTCCTGAGTA GGACAAATCC GCCGGGAGCG GATTTGAACG TTGCGAAGCA ACGGCCCGGA 180  
GGGTGGCGGG CAGGACGCCC GCCATAAACT GCCAGGCATC AAATTAAGCA GAAGGGGCCT 240  
35 CCCACCGCCC GTCCTGCGGG CGGTATTTGA CGGTCCGTAG TTTAATTCGT CTTCGCCATC 300  
CTGACGGATG GCCTTTTTGC GTTCTACAA ACTCTTTTGT TTATTTTCT AAATACATTC 360  
AAATATGGAC GTCTCATAAT TTTTAAAAAA TTCATTTGAC AAATGCTAAA ATTCTTGATT 420  
40 AATATTCCTCA ATTGTGAGCG CTCACAATTT ATCGATTTGA TTCTAGATTT GTTTTAACTA 480  
ATTAAAGGAG GAATAACATA TGGTTAACGC GTTGGAATTC GAGCTCACTA GTGTCGACCT 540  
GCAGGGTACC ATGGAAGCTT ACTCGAGGAT CCGCGGAAAG AAGAAGAAGA AGAAGAAAGC 600  
45 CCGAAAGGAA GCTGAGTTGG CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCTTGG 660  
GGCCTCTAAA CGGGTCTTGA GGGGTTTTTT GCTGAAAGGA GGAACCGCTC TTCACGCTCT 720  
50 TCACGC 726

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTTACTGATT GGAC

44

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GTCCTCCTGG TACCTACCTA AAACAAC

27

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 102 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTACCCGGCG

60

GACATTTATC ACACAGCAGC TGATGAGAAG TTTCTTCATC CA

102

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Met Asp Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro  
1 5 10 15

Gly Thr Tyr

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 84 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TATGGAAACT TTTCTCCAA AATATCTTCA TTATGATGAA GAACTTCTC ATCAGCTGCT 60

GTGTGATAAA TGTCCGCCGG GTAC 84

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 78 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCATAA TGAAGATATT 60

5 TTGGAGGAAA AGTTTCCA 78

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTCACGGATT GAAC 44

(2) INFORMATION FOR SEQ ID NO:66:

25 (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 38 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

40 GTGCTCCTGG TACCTACCTA AAACAGCACT GCACAGTG 38

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 84 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TATGGAAACT CTGCCTCCAA AATACCTGCA TTACGATCCG GAAACTGGTC ATCAGCTGCT 60

5 GTGTGATAAA TGTGCTCCGG GTAC 84

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 78 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT 60

25 TTGGAGGCAG AGTTTCCA 78

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 54 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

40 TATGGACCCA GAAACTGGTC ATCAGCTGCT GTGTGATAAA TGTGCTCCGG GTAC 54

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 48 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC TGGGTCCA

48

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 87 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

TATGAAAGAA ACTCTGCCTC CAAAATACCT GCATTACGAT CCGGAAACTG GTCATCAGCT

60

GCTGTGTGAT AAATGTGCTC CGGGTAC

87

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 81 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT

60

TTGGAGGCAG AGTTTCTTTC A

81

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 71 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

10 GTTCTCCTCA TATGAAACAT CATCACCATC ACCATCATGA AACTCTGCCT CCAAAATACC 60  
TGCATTACGA T 71

(2) INFORMATION FOR SEQ ID NO:74:

15

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 43 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GTTCTCCTCA TATGAAAGAA ACTCTGCCTC CAAATACCT GCA 43

30

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 76 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TACGCACTGG ATCCTTAATG ATGGTGATGG TGATGATGTA AGCAGCTTAT TTTCACGGAT 60

45

TGAACCTGAT TCCCTA 76

(2) INFORMATION FOR SEQ ID NO:76:

50

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 47 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GTTCTCCTCA TATGAAATAC CTGCATTACG ATCCGGAAAC TGGTCAT

47

10

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

25

GTTCTCCTAT TAATGAAATA TCTTCATTAT GATGAAGAAA CTT

43

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

35

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

TACGCACTGG ATCCTTATAA GCAGCTTATT TTTACTGATT

40

45

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA



5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

GTTCTCCTCA TATGGAAACT CTGCCTCCAA AATACCTGCA

40

(2) INFORMATION FOR SEQ ID NO:80:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TACGCACTGG ATCCTTATGT TGCATTTCTT TTCTGAATTA GCA

43

25

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

40

CCGGAACAG ATAATGAG

18

(2) INFORMATION FOR SEQ ID NO:82:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

5 GATCCTCATT ATCTGTTT

18

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CCGGAAACAG AGAAGCCACG CAAAAGTAAG

30

(2) INFORMATION FOR SEQ ID NO:84:

25 (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

40 GATCCTTACT TTTGCGTGGC TTCTCTGTTT

30

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 12 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

TATGTTAATG AG

12

5 (2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

20 GATCCTCATT AACA

14

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

TATGTTCCGG AAACAGTTAA G

21

(2) INFORMATION FOR SEQ ID NO:88:

40

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

45 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

GATCCTTAAC TGTTTCCGGA ACA

23

(2) INFORMATION FOR SEQ ID NO:89:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

TATGTTCCGG AAACAGTGAA TCAACTCAAA AATAAG

36

20 (2) INFORMATION FOR SEQ ID NO:90:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 38 base pairs  
(B) TYPE: nucleic acid  
25 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

35 GATCCTTATT TTTGAGTTGA TTCACTGTTT CCGGAACA

38

(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:  
40 (A) LENGTH: 100 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

50 CTAGCGACGA CGACGACAAA GAAACTCTGC CTCCAAAATA CCTGCATTAC GATCCGGAAA

60

CTGGTCATCA GCTGCTGTGT GATAAATGTG CTCCGGGTAC

100

(2) INFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 92 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

CCGGAGCACA TTTATCACAC AGCAGCTGAT GACCAGTTTC CGGATCGTAA TGCAGGTATT 60  
TTGGAGGCAG AGTTTCTTTG TCGTCGTCGT CG 92

(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 26 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

ACAAACACAA TCGATTTGAT ACTAGA 26

(2) INFORMATION FOR SEQ ID NO:94:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 50 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

TTTGTTTTAA CTAATTAAAG GAGGAATAAA ATATGAGAGG ATCGCATCAC

50

(2) INFORMATION FOR SEQ ID NO:95:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 50 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

CATCACCATC ACGAAACCTT CCCGCCGAAA TACCTGCACT ACGACGAAGA

50

(2) INFORMATION FOR SEQ ID NO:96:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 49 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

AACCTCCCAC CAGCTGCTGT GCGACAAATG CCCGCCGGGT ACCCAAACA

49

(2) INFORMATION FOR SEQ ID NO:97:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

TGTTTGGGTA CCCGGCGGGC ATTTGT

26

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CGCACAGCAG CTGGTGGGAG GTTTCTTCGT CGTAGTGCAG GTATTTCCGGC

50

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GGGAAGGTTT CGTGATGGTG ATGGTGATGC GATCCTCTCA TATTTTATT

49

(2) INFORMATION FOR SEQ ID NO:100:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

CCTCCTTTAA TTAGTTAAAA CAAATCTAGT ATCAAATCGA TTGTGTTTGT

50

(2) INFORMATION FOR SEQ ID NO:101:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 59 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

ACAAACACAA TCGATTTGAT ACTAGATTTG TTTTAACTAA TTAAAGGAGG AATAAAATG 59

(2) INFORMATION FOR SEQ ID NO:102:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 48 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

CTAATTAAAG GAGGAATAAA ATGAAAGAAA CTTTTCCTCC AAAATATC 48

(2) INFORMATION FOR SEQ ID NO:103:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 31 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C 31

(2) INFORMATION FOR SEQ ID NO:104:

- (i) SEQUENCE CHARACTERISTICS:



(A) LENGTH: 59 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

ACAAACACAA TCGATTTGAT ACTAGATTG TTTTAACTAA TTAAAGGAGG AATAAAATG 59

15

(2) INFORMATION FOR SEQ ID NO:105:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

30

CTAATTAAAG GAGGAATAAA ATGAAAAAAA AAGAACTTT TCCTCCAAAA TATC 54

(2) INFORMATION FOR SEQ ID NO:106:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35

40

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

TGTTTGGGTA CCCGGCGGAC ATTTATCACA C 31

(2) INFORMATION FOR SEQ ID NO:107:

50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

CAGCCCGGGT AAAATGGAAA CGTTTCCTCC AAAATATCTT CATT

44

(2) INFORMATION FOR SEQ ID NO:108:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

CGTTTCCATT TTACCCGGGC TGAGCGAGAG GCTCTTCTGC GTGT

44

(2) INFORMATION FOR SEQ ID NO:109:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 45 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

CGCTCAGCCC GGGTAAAATG GAAACGTTGC CTCCAAAATA CCTGC

45

(2) INFORMATION FOR SEQ ID NO:110:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

CCATTTTACC CGGGCTGAGC GAGAGGCTCT TCTGCGTGT

39

10

(2) INFORMATION FOR SEQ ID NO:111:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

GAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

25

(2) INFORMATION FOR SEQ ID NO:112:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

35

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

CAGCTGCAGC TAAGCAGCTT ATTTTCACGG ATTG

34

45

(2) INFORMATION FOR SEQ ID NO:113:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

AAAAATAAGC TGCTTAGCTG CAGCTGAACC AAAATC

36

(2) INFORMATION FOR SEQ ID NO:114:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

CAGCTGCAGC TAAGCAGCTT ATTTTACTG ATTGG

35

(2) INFORMATION FOR SEQ ID NO:115:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 102 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

CTAGAAGGAG GAATAACATA TGGAAACTTT TGCTCCAAAA TATCTTCATT ATGATGAAGA

60

AACTAGTCAT CAGCTGCTGT GTGATAAATG TCCGCCGGGT AC

102

(2) INFORMATION FOR SEQ ID NO:116:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 94 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

10

CCGGCGGACA TTTATCACAC AGCAGCTGAT GACTAGTTTC TTCATCATAA TGAAGATATT 60

TTGGAGCAAA AGTTTCCATA TGTTATTCCT CCTT 94

(2) INFORMATION FOR SEQ ID NO:117:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 62 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

CTAGAAGGAG GAATAACATA TGGAAACTTT TCCTGCTAAA TATCTTCATT ATGATGAAGA 60

30

AA 62

(2) INFORMATION FOR SEQ ID NO:118:

35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 62 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

CTAGTTTCTT CATCATAATG AAGATATTTA GCAGGAAAAG TTTCCATATG TTATTCCTCC 60

50

TT 62

(2) INFORMATION FOR SEQ ID NO:119:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 51 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

Tyr His Tyr Tyr Asp Gln Asn Gly Arg Met Cys Glu Glu Cys His Met  
1 5 10 15  
Cys Gln Pro Gly His Phe Leu Val Lys His Cys Lys Gln Pro Lys Arg  
20 25 30  
Asp Thr Val Cys His Lys Pro Cys Glu Pro Gly Val Thr Tyr Thr Asp  
35 40 45  
Asp Trp His  
50

(2) INFORMATION FOR SEQ ID NO:120:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2432 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS  
(B) LOCATION: 124..1326

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

ATCAAAGGCA GGGCATACTT CCTGTTGCCC AGACCTTATA TAAAACGTCA TGTTCGCCTG 60  
GGCAGCAGAG AAGCACCTAG CACTGGCCCA GCGGCTGCCG CCTGAGGTTT CCAGAGGACC 120  
ACA ATG AAC AAG TGG CTG TGC TGT GCA CTC CTG GTG TTC TTG GAC ATC 168  
Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile  
1 5 10 15

	ATT	GAA	TGG	ACA	ACC	CAG	GAA	ACC	TTT	CCT	CCA	AAA	TAC	TTG	CAT	TAT	216
	Ile	Glu	Trp	Thr	Thr	Gln	Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	Tyr	
					20					25						30	
5																	
	GAC	CCA	GAA	ACC	GGA	CGT	CAG	CTC	TTG	TGT	GAC	AAA	TGT	GCT	CCT	GGC	264
	Asp	Pro	Glu	Thr	Gly	Arg	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Ala	Pro	Gly	
					35				40					45			
10																	
	ACC	TAC	CTA	AAA	CAG	CAC	TGC	ACA	GTC	AGG	AGG	AAG	ACA	CTG	TGT	GTC	312
	Thr	Tyr	Leu	Lys	Gln	His	Cys	Thr	Val	Arg	Arg	Lys	Thr	Leu	Cys	Val	
				50				55					60				
15																	
	CCT	TGC	CCT	GAC	TAC	TCT	TAT	ACA	GAC	AGC	TGG	CAC	ACG	AGT	GAT	GAA	360
	Pro	Cys	Pro	Asp	Tyr	Ser	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	
		65					70					75					
20																	
	TGC	GTG	TAC	TGC	AGC	CCC	GTG	TGC	AAG	GAA	CTG	CAG	ACC	GTG	AAA	CAG	408
	Cys	Val	Tyr	Cys	Ser	Pro	Val	Cys	Lys	Glu	Leu	Gln	Thr	Val	Lys	Gln	
		80				85					90					95	
25																	
	GAG	TGC	AAC	CGC	ACC	CAC	AAC	CGA	GTG	TGC	GAA	TGT	GAG	GAA	GGG	CGC	456
	Glu	Cys	Asn	Arg	Thr	His	Asn	Arg	Val	Cys	Glu	Cys	Glu	Glu	Gly	Arg	
					100					105					110		
30																	
	TAC	CTG	GAG	CTC	GAA	TTC	TGC	TTG	AAG	CAC	CGG	AGC	TGT	CCC	CCA	GGC	504
	Tyr	Leu	Glu	Leu	Glu	Phe	Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	
					115				120					125			
35																	
	TTG	GGT	GTG	CTG	CAG	GCT	GGG	ACC	CCA	GAG	CGA	AAC	ACG	GTT	TGC	AAA	552
	Leu	Gly	Val	Leu	Gln	Ala	Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	
			130				135						140				
40																	
	AGA	TGT	CCG	GAT	GGG	TTC	TTC	TCA	GGT	GAG	ACG	TCA	TCG	AAA	GCA	CCC	600
	Arg	Cys	Pro	Asp	Gly	Phe	Phe	Ser	Gly	Glu	Thr	Ser	Ser	Lys	Ala	Pro	
			145				150					155					
45																	
	TGT	AGG	AAA	CAC	ACC	AAC	TGC	AGC	TCA	CTT	GGC	CTC	CTG	CTA	ATT	CAG	648
	Cys	Arg	Lys	His	Thr	Asn	Cys	Ser	Ser	Leu	Gly	Leu	Leu	Leu	Ile	Gln	
		160				165					170					175	
50																	
	AAA	GGA	AAT	GCA	ACA	CAT	GAC	AAT	GTA	TGT	TCC	GGA	AAC	AGA	GAA	GCA	696
	Lys	Gly	Asn	Ala	Thr	His	Asp	Asn	Val	Cys	Ser	Gly	Asn	Arg	Glu	Ala	
					180					185					190		
55																	
	ACT	CAA	AAT	TGT	GGA	ATA	GAT	GTC	ACC	CTG	TGC	GAA	GAG	GCA	TTC	TTC	744
	Thr	Gln	Asn	Cys	Gly	Ile	Asp	Val	Thr	Leu	Cys	Glu	Glu	Ala	Phe	Phe	
					195				200					205			
60																	
	AGG	TTT	GCT	GTG	CCT	ACC	AAG	ATT	ATA	CCG	AAT	TGG	CTG	AGT	GTT	CTG	792
	Arg	Phe	Ala	Val	Pro	Thr	Lys	Ile	Ile	Pro	Asn	Trp	Leu	Ser	Val	Leu	
			210					215					220				

	GTG GAC AGT TTG CCT GGG ACC AAA GTG AAT GCA GAG AGT GTA GAG AGG	840
	Val Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg	
	225 230 235	
5	ATA AAA CGG AGA CAC AGC TCG CAA GAG CAA ACT TTC CAG CTA CTT AAG	888
	Ile Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys	
	240 245 250 255	
10	CTG TGG AAG CAT CAA AAC AGA GAC CAG GAA ATG GTG AAG AAG ATC ATC	936
	Leu Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile	
	260 265 270	
15	CAA GAC ATT GAC CTC TGT GAA AGC AGT GTG CAA CGG CAT ATC GGC CAC	984
	Gln Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Ile Gly His	
	275 280 285	
20	GCG AAC CTC ACC ACA GAG CAG CTC CGC ATC TTG ATG GAG AGC TTG CCT	1032
	Ala Asn Leu Thr Thr Glu Gln Leu Arg Ile Leu Met Glu Ser Leu Pro	
	290 295 300	
25	GGG AAG AAG ATC AGC CCA GAC GAG ATT GAG AGA ACG AGA AAG ACC TGC	1080
	Gly Lys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys	
	305 310 315	
30	AAA CCC AGC GAG CAG CTC CTG AAG CTA CTG AGC TTG TGG AGG ATC AAA	1128
	Lys Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys	
	320 325 330 335	
35	AAT GGA GAC CAA GAC ACC TTG AAG GGC CTG ATG TAC GCA CTC AAG CAC	1176
	Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His	
	340 345 350	
40	TTG AAA GCA TAC CAC TTT CCC AAA ACC GTC ACC CAC AGT CTG AGG AAG	1224
	Leu Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys	
	355 360 365	
45	ACC ATC AGG TTC TTG CAC AGC TTC ACC ATG TAC CGA TTG TAT CAG AAA	1272
	Thr Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys	
	370 375 380	
50	CTC TTT CTA GAA ATG ATA GGG AAT CAG GTT CAA TCA GTG AAG ATA AGC	1320
	Leu Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser	
	385 390 395	
55	TGC TTA TAGTTAGGAA TGGTCACTGG GCTGTTTCTT CAGGATGGGC CAACACTGAT	1376
	Cys Leu	
	400	
60	GGAGCAGATG GCTGCTTCTC CGGCTCTTGA AATGGCAGTT GATTCCCTTC TCATCAGTTG	1436
	GTGGGAATGA AGATCCTCCA GCCCAACACA CACACTGGGG AGTCTGAGTC AGGAGAGTGA	1496



GGCAGGCTAT TTGATAATTG TGCAAAGCTG CCAGGTGTAC ACCTAGAAAG TCAAGCACCC 1556  
 TGAGAAAGAG GATATTTTAA TAACCTCAAA CATAGGCCCT TTCCTTCCTC TCCTTATGGA 1616  
 5 TGAGTACTCA GAAGGCTTCT ACTATCTTCT GTGTCATCCC TAGATGAAGG CCTCTTTTAT 1676  
 TTATTTTTTT ATTCTTTTTT TCGGAGCTGG GGACCGAACC CAGGGCCTTG CGCTTGCGAG 1736  
 10 GCAAGTGCTC TACCACTGAG CTAAATCTCC AACCCTGAA GGCCTCTTTC TTTCTGCCTC 1796  
 TGATAGTCTA TGACATTCTT TTTTCTACAA TTCGTATCAG GTGCACGAGC CTTATCCCAT 1856  
 TTGTAGGTTT CTAGGCAAGT TGACCGTTAG CTATTTTTC CTCTGAAGAT TTGATTCGAG 1916  
 15 TTGCAGACTT GGCTAGACAA GCAGGGGTAG GTTATGGTAG TTTATTTAAC AGACTGCCAC 1976  
 CAGGAGTCCA GTGTTTCTTG TTCCTCTGTA GTTGTACCTA AGCTGACTCC AAGTACATTT 2036  
 20 AGTATGAAAA ATAATCAACA AATTTTATTC CTTCTATCAA CATTGGCTAG CTTTGTTTCA 2096  
 GGGCACTAAA AGAACTACT ATATGGAGAA AGAATTGATA TTGCCCCCAA CGTTCAACAA 2156  
 CCCAATAGTT TATCCAGCTG TCATGCCTGG TTCAGTGTCT ACTGACTATG CGCCCTCTTA 2216  
 25 TTACTGCATG CAGTAATTCA ACTGGAAATA GTAATAATAA TAATAGAAAT AAAATCTAGA 2276  
 CTCCATTGGA TCTCTCTGAA TATGGGAATA TCTAACTTAA GAAGCTTTGA GATTCAGTT 2336  
 GTGTAAAGG CTTTATATTA AAAGCTGATG CTCTTCTGTA AAAGTTACTA ATATATCTGT 2396  
 30 AAGACTATTA CAGTATTGCT ATTTATATCC ATCCAG 2432

(2) INFORMATION FOR SEQ ID NO:121:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 401 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile  
 1 5 10 15  
 Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp  
 20 25 30

	Pro	Glu	Thr	Gly	Arg	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Ala	Pro	Gly	Thr	
				35				40					45				
5	Tyr	Leu	Lys	Gln	His	Cys	Thr	Val	Arg	Arg	Lys	Thr	Leu	Cys	Val	Pro	
		50					55					60					
	Cys	Pro	Asp	Tyr	Ser	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	
	65					70					75					80	
10	Val	Tyr	Cys	Ser	Pro	Val	Cys	Lys	Glu	Leu	Gln	Thr	Val	Lys	Gln	Glu	
					85					90					95		
	Cys	Asn	Arg	Thr	His	Asn	Arg	Val	Cys	Glu	Cys	Glu	Glu	Gly	Arg	Tyr	
15				100					105					110			
	Leu	Glu	Leu	Glu	Phe	Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Leu	
			115					120					125				
20	Gly	Val	Leu	Gln	Ala	Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg	
		130					135					140					
	Cys	Pro	Asp	Gly	Phe	Phe	Ser	Gly	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	
	145					150					155					160	
25	Arg	Lys	His	Thr	Asn	Cys	Ser	Ser	Leu	Gly	Leu	Leu	Leu	Ile	Gln	Lys	
					165					170					175		
	Gly	Asn	Ala	Thr	His	Asp	Asn	Val	Cys	Ser	Gly	Asn	Arg	Glu	Ala	Thr	
30				180					185					190			
	Gln	Asn	Cys	Gly	Ile	Asp	Val	Thr	Leu	Cys	Glu	Glu	Ala	Phe	Phe	Arg	
		195						200					205				
35	Phe	Ala	Val	Pro	Thr	Lys	Ile	Ile	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val	
		210					215					220					
	Asp	Ser	Leu	Pro	Gly	Thr	Lys	Val	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile	
	225					230					235					240	
40	Lys	Arg	Arg	His	Ser	Ser	Gln	Glu	Gln	Thr	Phe	Gln	Leu	Leu	Lys	Leu	
					245					250					255		
	Trp	Lys	His	Gln	Asn	Arg	Asp	Gln	Glu	Met	Val	Lys	Lys	Ile	Ile	Gln	
45				260					265					270			
	Asp	Ile	Asp	Leu	Cys	Glu	Ser	Ser	Val	Gln	Arg	His	Ile	Gly	His	Ala	
		275						280					285				
50	Asn	Leu	Thr	Thr	Glu	Gln	Leu	Arg	Ile	Leu	Met	Glu	Ser	Leu	Pro	Gly	
		290					295					300					

ys Lys Ile Ser Pro Asp Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys  
305 310 315 320

Pro Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn  
325 330 335

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu  
340 345 350

Lys Ala Tyr His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr  
355 360 365

Ile Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu  
370 375 380

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys  
385 390 395 400

Leu

(2) INFORMATION FOR SEQ ID NO:122:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1324 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 90..1292

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

40	CCTTATATAA ACGTCATGAT TGCCTGGGCT GCAGAGACGC ACCTAGCACT GACCCAGCGG	60
	CTGCCTCCTG AGGTTTCCCG AGGACCACA ATG AAC AAG TGG CTG TGC TGC GCA	113
	Met Asn Lys Trp Leu Cys Cys Ala	
	1 5	
45		
	CTC CTG GTG CTC CTG GAC ATC ATT GAA TGG ACA ACC CAG GAA ACC CTT	161
	Leu Leu Val Leu Leu Asp Ile Ile Glu Trp Thr Thr Gln Glu Thr Leu	
	10 15 20	
50		
	CCT CCA AAG TAC TTG CAT TAT GAC CCA GAA ACT GGT CAT CAG CTC CTG	209
	Pro Pro Lys Tyr Leu His Tyr Asp Pro Glu Thr Gly His Gln Leu Leu	
	25 30 35 40	

5	AGG	AGG	AAG	ACA	TTG	TGT	GTC	CCT	TGC	CCT	GAC	CAC	TCT	TAT	ACG	GAC	305
	Arg	Arg	Lys	Thr	Leu	Cys	Val	Pro	Cys	Pro	Asp	His	Ser	Tyr	Thr	Asp	
				60					65					70			
10	AGC	TGG	CAC	ACC	AGT	GAT	GAG	TGT	GTG	TAT	TGC	AGC	CCA	GTG	TGC	AAG	353
	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	Val	Tyr	Cys	Ser	Pro	Val	Cys	Lys	
			75					80					85				
15	GAA	CTG	CAG	TCC	GTG	AAG	CAG	GAG	TGC	AAC	CGC	ACC	CAC	AAC	CGA	GTG	401
	Glu	Leu	Gln	Ser	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His	Asn	Arg	Val	
		90					95					100					
20	TGT	GAG	TGT	GAG	GAA	GGG	CGT	TAC	CTG	GAG	ATC	GAA	TTC	TGC	TTG	AAG	449
	Cys	Glu	Cys	Glu	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe	Cys	Leu	Lys	
	105					110					115					120	
25	CAC	CGG	AGC	TGT	CCC	CCG	GGC	TCC	GGC	GTG	GTG	CAA	GCT	GGA	ACC	CCA	497
	His	Arg	Ser	Cys	Pro	Pro	Gly	Ser	Gly	Val	Val	Gln	Ala	Gly	Thr	Pro	
				125						130					135		
30	GAG	CGA	AAC	ACA	GTT	TGC	AAA	AAA	TGT	CCA	GAT	GGG	TTC	TTC	TCA	GGT	545
	Glu	Arg	Asn	Thr	Val	Cys	Lys	Lys	Cys	Pro	Asp	Gly	Phe	Phe	Ser	Gly	
				140					145					150			
35	GAG	ACT	TCA	TCG	AAA	GCA	CCC	TGT	ATA	AAA	CAC	ACG	AAC	TGC	AGC	ACA	593
	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	Ile	Lys	His	Thr	Asn	Cys	Ser	Thr	
			155					160					165				
40	TTT	GGC	CTC	CTG	CTA	ATT	CAG	AAA	GGA	AAT	GCA	ACA	CAT	GAC	AAC	GTG	641
	Phe	Gly	Leu	Leu	Leu	Ile	Gln	Lys	Gly	Asn	Ala	Thr	His	Asp	Asn	Val	
		170					175					180					
45	TGT	TCC	GGA	AAC	AGA	GAA	GCC	ACG	CAA	AAG	TGT	GGA	ATA	GAT	GTC	ACC	689
	Cys	Ser	Gly	Asn	Arg	Glu	Ala	Thr	Gln	Lys	Cys	Gly	Ile	Asp	Val	Thr	
	185					190					195					200	
50	CTG	TGT	GAA	GAG	GCC	TTC	TTC	AGG	TTT	GCT	GTT	CCT	ACC	AAG	ATT	ATA	737
	Leu	Cys	Glu	Glu	Ala	Phe	Phe	Arg	Phe	Ala	Val	Pro	Thr	Lys	Ile	Ile	
				205						210					215		
55	CCA	AAT	TGG	CTG	AGT	GTT	TTG	GTG	GAC	AGT	TTG	CCT	GGG	ACC	AAA	GTG	785
	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val	Asp	Ser	Leu	Pro	Gly	Thr	Lys	Val	
				220					225					230			
60	AAT	GCC	GAG	AGT	GTA	GAG	AGG	ATA	AAA	CGG	AGA	CAC	AGC	TCA	CAA	GAG	833
	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile	Lys	Arg	Arg	His	Ser	Ser	Gln	Glu	
			235					240					245				

	CAA ACC TTC CAG CTG CTG AAG CTG TGG AAA CAT CAA AAC AGA GAC CAG	881
	Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Arg Asp Gln	
	250 255 260	
5	GAA ATG GTG AAG AAG ATC ATC CAA GAC ATT GAC CTC TGT GAA AGC AGC	929
	Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Ser Ser	
	265 270 275 280	
10	GTG CAG CGG CAT CTC GGC CAC TCG AAC CTC ACC ACA GAG CAG CTT CTT	977
	Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu Gln Leu Leu	
	285 290 295	
15	GCC TTG ATG GAG AGC CTG CCT GGG AAG AAG ATC AGC CCA GAA GAG ATT	1025
	Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro Glu Glu Ile	
	300 305 310	
20	GAG AGA ACG AGA AAG ACC TGC AAA TCG AGC GAG CAG CTC CTG AAG CTA	1073
	Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu Leu Lys Leu	
	315 320 325	
25	CTC AGT TTA TGG AGG ATC AAA AAT GGT GAC CAA GAC ACC TTG AAG GGC	1121
	Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly	
	330 335 340	
30	CTG ATG TAT GCC CTC AAG CAC TTG AAA ACA TCC CAC TTT CCC AAA ACT	1169
	Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe Pro Lys Thr	
	345 350 355 360	
35	GTC ACC CAC AGT CTG AGG AAG ACC ATG AGG TTC CTG CAC AGC TTC ACA	1217
	Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His Ser Phe Thr	
	365 370 375	
40	ATG TAC AGA CTG TAT CAG AAG CTC TTT TTA GAA ATG ATA GGG AAT CAG	1265
	Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn Gln	
	380 385 390	
45	GTT CAA TCC GTG AAA ATA AGC TGC TTA TAACTAGGAA TGGTCACTGG	1312
	Val Gln Ser Val Lys Ile Ser Cys Leu	
	395 400	
50	GCTGTTTCTT CA	1324

(2) INFORMATION FOR SEQ ID NO:123:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 401 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

5	Met	Asn	Lys	Trp	Leu	Cys	Cys	Ala	Leu	Leu	Val	Leu	Leu	Asp	Ile	Ile	1	5	10	15
	Glu	Trp	Thr	Thr	Gln	Glu	Thr	Leu	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp	20	25	30	
10	Pro	Glu	Thr	Gly	His	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Ala	Pro	Gly	Thr	35	40	45	
	Tyr	Leu	Lys	Gln	His	Cys	Thr	Val	Arg	Arg	Lys	Thr	Leu	Cys	Val	Pro	50	55	60	
15	Cys	Pro	Asp	His	Ser	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	65	70	75	80
	Val	Tyr	Cys	Ser	Pro	Val	Cys	Lys	Glu	Leu	Gln	Ser	Val	Lys	Gln	Glu	85	90	95	
20	Cys	Asn	Arg	Thr	His	Asn	Arg	Val	Cys	Glu	Cys	Glu	Glu	Gly	Arg	Tyr	100	105	110	
	Leu	Glu	Ile	Glu	Phe	Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Ser	115	120	125	
25	Gly	Val	Val	Gln	Ala	Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Lys	130	135	140	
30	Cys	Pro	Asp	Gly	Phe	Phe	Ser	Gly	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	145	150	155	160
	Ile	Lys	His	Thr	Asn	Cys	Ser	Thr	Phe	Gly	Leu	Leu	Leu	Ile	Gln	Lys	165	170	175	
35	Gly	Asn	Ala	Thr	His	Asp	Asn	Val	Cys	Ser	Gly	Asn	Arg	Glu	Ala	Thr	180	185	190	
40	Gln	Lys	Cys	Gly	Ile	Asp	Val	Thr	Leu	Cys	Glu	Glu	Ala	Phe	Phe	Arg	195	200	205	
	Phe	Ala	Val	Pro	Thr	Lys	Ile	Ile	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val	210	215	220	
45	Asp	Ser	Leu	Pro	Gly	Thr	Lys	Val	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile	225	230	235	240
	Lys	Arg	Arg	His	Ser	Ser	Gln	Glu	Gln	Thr	Phe	Gln	Leu	Leu	Lys	Leu	245	250	255	
50	Trp	Lys	His	Gln	Asn	Arg	Asp	Gln	Glu	Met	Val	Lys	Lys	Ile	Ile	Gln	260	265	270	

Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Leu Gly His Ser  
275 280 285

5 Asn Leu Thr Thr Glu Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly  
290 295 300

Lys Lys Ile Ser Pro Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys  
305 310 315 320

10 Ser Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn  
325 330 335

15 Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu  
340 345 350

Lys Thr Ser His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr  
355 360 365

20 Met Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu  
370 375 380

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys  
385 390 395 400

25 Leu

30 (2) INFORMATION FOR SEQ ID NO:124:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1355 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- 40 (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 94..1296

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

GTATATATAA CGTGATGAGC GTACGGGTGC GGAGACGCAC CGGAGCGCTC GCCCAGCCGC 60

50 CGCTCCAAGC CCCTGAGGTT TCCGGGGACC ACA ATG AAC AAG TTG CTG TGC TGC 114  
Met Asn Lys Leu Leu Cys Cys

	GCG CTC GTG TTT CTG GAC ATC TCC ATT AAG TGG ACC ACC CAG GAA ACG	162
	Ala Leu Val Phe Leu Asp Ile Ser Ile Lys Trp Thr Thr Gln Glu Thr	
	10 15 20	
5	TTT CCT CCA AAG TAC CTT CAT TAT GAC GAA GAA ACC TCT CAT CAG CTG	210
	Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His Gln Leu	
	25 30 35	
10	TTG TGT GAC AAA TGT CCT CCT GGT ACC TAC CTA AAA CAA CAC TGT ACA	258
	Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His Cys Thr	
	40 45 50 55	
15	GCA AAG TGG AAG ACC GTG TGC GCC CCT TGC CCT GAC CAC TAC TAC ACA	306
	Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr Tyr Thr	
	60 65 70	
20	GAC AGC TGG CAC ACC AGT GAC GAG TGT CTA TAC TGC AGC CCC GTG TGC	354
	Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro Val Cys	
	75 80 85	
25	AAG GAG CTG CAG TAC GTC AAG CAG GAG TGC AAT CGC ACC CAC AAC CGC	402
	Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His Asn Arg	
	90 95 100	
30	GTG TGC GAA TGC AAG GAA GGG CGC TAC CTT GAG ATA GAG TTC TGC TTG	450
	Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe Cys Leu	
	105 110 115	
35	AAA CAT AGG AGC TGC CCT CCT GGA TTT GGA GTG GTG CAA GCT GGA ACC	498
	Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala Gly Thr	
	120 125 130 135	
40	CCA GAG CGA AAT ACA GTT TGC AAA AGA TGT CCA GAT GGG TTC TTC TCA	546
	Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe Phe Ser	
	140 145 150	
45	AAT GAG ACG TCA TCT AAA GCA CCC TGT AGA AAA CAC ACA AAT TGC AGT	594
	Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn Cys Ser	
	155 160 165	
50	GTC TTT GGT CTC CTG CTA ACT CAG AAA GGA AAT GCA ACA CAC GAC AAC	642
	Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His Asp Asn	
	170 175 180	
55	ATA TGT TCC GGA AAC AGT GAA TCA ACT CAA AAA TGT GGA ATA GAT GTT	690
	Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile Asp Val	
	185 190 195	
60	ACC CTG TGT GAG GAG GCA TTC TTC AGG TTT GCT GTT CCT ACA AAG TTT	738
	Thr Leu Cys Glu Glu Ala Phe Phe Arg Phe Ala Val Pro Thr Lys Phe	
	200 205 210 215	



	ACG CCT AAC TGG CTT AGT GTC TTG GTA GAC AAT TTG CCT GGC ACC AAA	786
	Thr Pro Asn Trp Leu Ser Val Leu Val Asp Asn Leu Pro Gly Thr Lys	
	220 225 230	
5	GTA AAC GCA GAG AGT GTA GAG AGG ATA AAA CGG CAA CAC AGC TCA CAA	834
	Val Asn Ala Glu Ser Val Glu Arg Ile Lys Arg Gln His Ser Ser Gln	
	235 240 245	
10	GAA CAG ACT TTC CAG CTG CTG AAG TTA TGG AAA CAT CAA AAC AAA GCC	882
	Glu Gln Thr Phe Gln Leu Leu Lys Leu Trp Lys His Gln Asn Lys Ala	
	250 255 260	
15	CAA GAT ATA GTC AAG AAG ATC ATC CAA GAT ATT GAC CTC TGT GAA AAC	930
	Gln Asp Ile Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys Glu Asn	
	265 270 275	
20	AGC GTG CAG CGG CAC ATT GGA CAT GCT AAC CTC ACC TTC GAG CAG CTT	978
	Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr Phe Glu Gln Leu	
	280 285 290 295	
25	CGT AGC TTG ATG GAA AGC TTA CCG GGA AAG AAA GTG GGA GCA GAA GAC	1026
	Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp	
	300 305 310	
30	ATT GAA AAA ACA ATA AAG GCA TGC AAA CCC AGT GAC CAG ATC CTG AAG	1074
	Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys	
	315 320 325	
35	CTG CTC AGT TTG TGG CGA ATA AAA AAT GGC GAC CAA GAC ACC TTG AAG	1122
	Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys	
	330 335 340	
40	GGC CTA ATG CAC GCA CTA AAG CAC TCA AAG ACG TAC CAC TTT CCC AAA	1170
	Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Lys	
	345 350 355	
45	ACT GTC ACT CAG AGT CTA AAG AAG ACC ATC AGG TTC CTT CAC AGC TTC	1218
	Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His Ser Phe	
	360 365 370 375	
50	ACA ATG TAC AAA TTG TAT CAG AAG TTA TTT TTA GAA ATG ATA GGT AAC	1266
	Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly Asn	
	380 385 390	
55	CAG GTC CAA TCA GTA AAA ATA AGC TGC TTA TAACTGGAAA TGGCCATTGA	1316
	Gln Val Gln Ser Val Lys Ile Ser Cys Leu	
	395 400	
60	GCTGTTTCCT CACAATTGGC GAGATCCCAT GGATGATAA	1355

(2) INFORMATION FOR SEQ ID NO:125:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 401 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

```

10  Met Asn Lys Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser Ile
    1          5          10          15

    Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
        20          25          30

15  Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr
    35          40          45

    Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro
    50          55          60

20  Cys Pro Asp His Tyr Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
    65          70          75          80

25  Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu
        85          90          95

    Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr
        100          105          110

30  Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe
    115          120          125

    Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
    130          135          140

35  Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys
    145          150          155          160

40  Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys
        165          170          175

    Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr
        180          185          190

45  Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
    195          200          205

    Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val
    210          215          220

50  Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
    225          230          235          240

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Leu

(ii) MOLECULE TYPE: protein

Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys  
1 5 10 15

- 195 -

Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro  
20 25 30

Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala  
35 40 45

Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys  
50 55 60

Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr  
65 70 75 80

Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn  
85 90 95

Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His  
100 105 110

Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly  
115 120 125

Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys  
130 135

(2) INFORMATION FOR SEQ ID NO:127:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 48 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

CCGGCGGACA TTTATCACAC AGCAGCTGAT GAGAAGTTTC TTCATCCA

48

(2) INFORMATION FOR SEQ ID NO:128:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 219 amino acids  
(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

Met	Leu	Gly	Ile	Trp	Thr	Leu	Leu	Pro	Leu	Val	Leu	Thr	Ser	Val	Ala	1	5	10	15
Arg	Leu	Ser	Ser	Lys	Ser	Val	Asn	Ala	Gln	Val	Thr	Asp	Ile	Asn	Ser	20	25	30	
Lys	Gly	Leu	Glu	Leu	Arg	Lys	Thr	Val	Thr	Thr	Val	Glu	Thr	Gln	Asn	35	40	45	
Leu	Glu	Gly	Leu	His	His	Asp	Gly	Gln	Phe	Cys	His	Lys	Pro	Cys	Pro	50	55	60	
Pro	Gly	Glu	Arg	Lys	Ala	Arg	Asp	Cys	Thr	Val	Asn	Gly	Asp	Glu	Pro	65	70	75	80
Asp	Cys	Val	Pro	Cys	Gln	Glu	Gly	Lys	Glu	Tyr	Thr	Asp	Lys	Ala	His	85	90	95	
Phe	Ser	Ser	Lys	Cys	Arg	Arg	Cys	Arg	Leu	Cys	Asp	Glu	Gly	His	Gly	100	105	110	
Leu	Glu	Val	Glu	Ile	Asn	Cys	Thr	Arg	Thr	Gln	Asn	Thr	Lys	Cys	Arg	115	120	125	
Cys	Lys	Pro	Asn	Phe	Phe	Cys	Asn	Ser	Thr	Val	Cys	Glu	His	Cys	Asp	130	135	140	
Pro	Cys	Thr	Lys	Cys	Glu	His	Gly	Ile	Ile	Lys	Glu	Cys	Thr	Leu	Thr	145	150	155	160
Ser	Asn	Thr	Lys	Cys	Lys	Glu	Glu	Gly	Ser	Arg	Ser	Asn	Leu	Gly	Trp	165	170	175	
Leu	Cys	Leu	Leu	Leu	Leu	Pro	Ile	Pro	Leu	Ile	Val	Trp	Val	Lys	Arg	180	185	190	
Lys	Glu	Val	Gln	Lys	Thr	Cys	Arg	Lys	His	Arg	Lys	Glu	Asn	Gln	Gly	195	200	205	
Ser	His	Glu	Ser	Pro	Thr	Leu	Asn	Pro	Glu	Thr	210	215							

(2) INFORMATION FOR SEQ ID NO:129:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 280 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:129:

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu  
1 5 10 15  
Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro  
20 25 30  
His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys  
35 40 45  
Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys  
50 55 60  
Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp  
65 70 75 80  
Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu  
85 90 95  
Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val  
100 105 110  
Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg  
115 120 125  
Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe  
130 135 140  
Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu  
145 150 155 160  
Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu  
165 170 175  
Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr  
180 185 190  
Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser  
195 200 205

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu  
210 215 220

5 Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys  
225 230 235 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu  
245 250 255

10 Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser  
260 265 270

15 Phe Ser Pro Thr Pro Gly Phe Thr  
275 280

(2) INFORMATION FOR SEQ ID NO:130:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 207 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

Met Leu Arg Leu Ile Ala Leu Leu Val Cys Val Val Tyr Val Tyr Gly  
1 5 10 15

35 Asp Asp Val Pro Tyr Ser Ser Asn Gln Gly Lys Cys Gly Gly His Asp  
20 25 30

Tyr Glu Lys Asp Gly Leu Cys Cys Ala Ser Cys His Pro Gly Phe Tyr  
35 40 45

40 Ala Ser Arg Leu Cys Gly Pro Gly Ser Asn Thr Val Cys Ser Pro Cys  
50 55 60

45 Glu Asp Gly Thr Phe Thr Ala Ser Thr Asn His Ala Pro Ala Cys Val  
65 70 75 80

Ser Cys Arg Gly Pro Cys Thr Gly His Leu Ser Glu Ser Gln Pro Cys  
85 90 95

Asp Arg Thr His Asp Arg Val Cys Asn Cys Ser Thr Gly Asn Tyr Cys  
 100 105 110

Leu Leu Lys Gly Gln Asn Gly Cys Arg Ile Cys Ala Pro Gln Thr Lys  
 115 120 125

Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Ala Gly Asp Thr  
 130 135 140

Leu Cys Glu Lys Cys Pro Pro His Thr Tyr Ser Asp Ser Leu Ser Pro  
 145 150 155 160

Thr Glu Arg Cys Gly Thr Ser Phe Asn Tyr Ile Ser Val Gly Phe Asn  
 165 170 175

Leu Tyr Pro Val Asn Glu Thr Ser Cys Thr Thr Thr Ala Gly His Asn  
 180 185 190

Glu Val Ile Lys Thr Lys Glu Phe Thr Val Thr Leu Asn Tyr Thr  
 195 200 205

(2) INFORMATION FOR SEQ ID NO:131:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 227 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu  
 1 5 10 15

Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr  
 20 25 30

Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln  
 35 40 45

Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys  
 50 55 60

Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp  
 65 70 75 80

Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys  
 85 90 95



Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg  
100 105 110

5 Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu  
115 120 125

Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg  
130 135 140

10 Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val  
145 150 155 160

Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr  
165 170 175

15 Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly  
180 185 190

20 Asn Ala Ser Arg Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser  
195 200 205

Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser  
210 215 220

25 Gln His Thr  
225

(2) INFORMATION FOR SEQ ID NO:132:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 197 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:

Met Val Ser Leu Pro Arg Leu Cys Ala Leu Trp Gly Cys Leu Leu Thr  
1 5 10 15

Ala Val His Leu Gly Gln Cys Val Thr Cys Ser Asp Lys Gln Tyr Leu  
20 25 30

50 His Asp Gly Gln Cys Cys Asp Leu Cys Gln Pro Gly Ser Arg Leu Thr  
35 40 45

Ser His Cys Thr Ala Leu Glu Lys Thr Gln Cys His Pro Cys Asp Ser  
50 55 60

5 Gly Glu Phe Ser Ala Gln Trp Asn Arg Glu Ile Arg Cys His Gln His  
65 70 75 80

Arg His Cys Glu Pro Asn Gln Gly Leu Arg Val Lys Lys Glu Gly Thr  
85 90 95

10 Ala Glu Ser Asp Thr Val Cys Thr Cys Lys Glu Gly Gln His Cys Thr  
100 105 110

15 Ser Lys Asp Cys Glu Ala Cys Ala Gln His Thr Pro Cys Ile Pro Gly  
115 120 125

Phe Gly Val Met Glu Met Ala Thr Glu Thr Thr Asp Thr Val Cys His  
130 135 140

20 Pro Cys Pro Val Gly Phe Phe Ser Asn Gln Ser Ser Leu Phe Glu Lys  
145 150 155 160

Cys Tyr Pro Trp Thr Ser Cys Glu Asp Lys Asn Leu Glu Val Leu Gln  
165 170 175

25 Lys Gly Thr Ser Gln Thr Asn Val Ile Cys Gly Leu Lys Ser Arg Met  
180 185 190

Arg Ala Leu Leu Val  
195

(2) INFORMATION FOR SEQ ID NO:133:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 208 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:

45 Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Phe Leu Asp Ile Ile  
1 5 10 15

Glu Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp  
20 25 30

50 Pro Glu Thr Gly Arg Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr  
35 40 45

Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro  
50 55 60

5 Cys Pro Asp Tyr Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys  
65 70 75 80

Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Thr Val Lys Gln Glu  
85 90 95

10 Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr  
100 105 110

15 Leu Glu Leu Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Leu  
115 120 125

Gly Val Leu Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg  
130 135 140

20 Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys  
145 150 155 160

Arg Lys His Thr Asn Cys Ser Ser Leu Gly Leu Leu Leu Ile Gln Lys  
165 170 175

25 Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr  
180 185 190

Gln Asn Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg  
195 200 205

(2) INFORMATION FOR SEQ ID NO:134:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 224 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:

Met Gly Ala Gly Ala Thr Gly Arg Ala Met Asp Gly Pro Arg Leu Leu  
1 5 10 15

Leu Leu Leu Leu Leu Gly Val Ser Leu Gly Gly Ala Lys Glu Ala Cys  
20 25 30

Pro Thr Gly Leu Tyr Thr His Ser Gly Glu Cys Cys Lys Ala Cys Asn  
35 40 45

Leu Gly Glu Gly Val Ala Gln Pro Cys Gly Ala Asn Gln Thr Val Cys  
50 55 60

5 Glu Pro Cys Leu Asp Ser Val Thr Phe Ser Asp Val Val Ser Ala Thr  
65 70 75 80

Glu Pro Cys Lys Pro Cys Thr Glu Cys Val Gly Leu Gln Ser Met Ser  
85 90 95

10 Ala Pro Cys Val Glu Ala Asp Asp Ala Val Cys Arg Cys Ala Tyr Gly  
100 105 110

15 Tyr Tyr Gln Asp Glu Thr Thr Gly Arg Cys Glu Ala Cys Arg Val Cys  
115 120 125

Glu Ala Gly Ser Gly Leu Val Phe Ser Cys Gln Asp Lys Gln Asn Thr  
130 135 140

20 Val Cys Glu Glu Cys Pro Asp Gly Thr Tyr Ser Asp Glu Ala Asn His  
145 150 155 160

Val Asp Pro Cys Leu Pro Cys Thr Val Cys Glu Asp Thr Glu Arg Gln  
165 170 175

25 Leu Arg Glu Cys Thr Arg Trp Ala Asp Ala Glu Cys Glu Glu Ile Pro  
180 185 190

30 Gly Arg Trp Ile Thr Arg Ser Thr Pro Pro Glu Gly Ser Asp Ser Thr  
195 200 205

Ala Pro Ser Thr Gln Glu Pro Glu Ala Pro Pro Glu Gln Asp Leu Ile  
210 215 220

(2) INFORMATION FOR SEQ ID NO:135:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 205 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:

Met Tyr Val Trp Val Gln Gln Pro Thr Ala Phe Leu Leu Leu Gly Leu  
1 5 10 15

Ser Leu Gly Val Thr Val Lys Leu Asn Cys Val Lys Asp Thr Tyr Pro  
20 25 30

Ser Gly His Lys Cys Cys Arg Glu Cys Gln Pro Gly His Gly Met Val  
35 40 45

Ser Arg Cys Asp His Thr Arg Asp Thr Val Cys His Pro Cys Glu Pro  
5 50 55 60

Gly Phe Tyr Asn Glu Ala Val Asn Tyr Asp Thr Cys Lys Gln Cys Thr  
65 70 75 80

Gln Cys Asn His Arg Ser Gly Ser Glu Leu Lys Gln Asn Cys Thr Pro  
85 90 95

Thr Glu Asp Thr Val Cys Gln Cys Arg Pro Gly Thr Gln Pro Arg Gln  
15 100 105 110

Asp Ser Ser His Lys Leu Gly Val Asp Cys Val Pro Cys Pro Pro Gly  
115 120 125

His Phe Ser Pro Gly Ser Asn Gln Ala Cys Lys Pro Trp Thr Asn Cys  
20 130 135 140

Thr Leu Ser Gly Lys Gln Ile Arg His Pro Ala Ser Asn Ser Leu Asp  
145 150 155 160

Thr Val Cys Glu Asp Arg Ser Leu Leu Ala Thr Leu Leu Trp Glu Thr  
165 170 175

Gln Arg Thr Thr Phe Arg Pro Thr Thr Val Pro Ser Thr Thr Val Trp  
30 180 185 190

Pro Arg Thr Ser Gln Leu Pro Ser Thr Pro Thr Leu Val  
195 200 205

(2) INFORMATION FOR SEQ ID NO:136:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 191 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:

Met Gly Asn Asn Cys Tyr Asn Val Val Val Ile Val Leu Leu Leu Val  
1 5 10 15

Gly Cys Glu Lys Val Gly Ala Val Gln Asn Ser Cys Asp Asn Cys Gln  
20 25 30

Pro Gly Thr Phe Cys Arg Lys Tyr Asn Pro Val Cys Lys Ser Cys Pro  
5 35 40 45

Pro Ser Thr Phe Ser Ser Ile Gly Gly Gln Pro Asn Cys Asn Ile Cys  
50 55 60

Arg Val Cys Ala Gly Tyr Phe Arg Phe Lys Lys Phe Cys Ser Ser Thr  
10 65 70 75 80

His Asn Ala Glu Cys Glu Cys Ile Glu Gly Phe His Cys Leu Gly Pro  
15 85 90 95

Gln Cys Thr Arg Cys Glu Lys Asp Cys Arg Pro Gly Gln Glu Leu Thr  
100 105 110

Lys Gln Gly Cys Lys Thr Cys Ser Leu Gly Thr Phe Asn Asp Gln Asn  
20 115 120 125

Gly Thr Gly Val Cys Arg Pro Trp Thr Asn Cys Ser Leu Asp Gly Arg  
130 135 140

Ser Val Leu Lys Thr Gly Thr Thr Glu Lys Asp Val Val Cys Gly Pro  
25 145 150 155 160

Pro Val Val Ser Phe Ser Pro Ser Thr Thr Ile Ser Val Thr Pro Glu  
30 165 170 175

Gly Gly Pro Gly Gly His Ser Leu Gln Val Leu Thr Leu Phe Leu  
180 185 190

(2) INFORMATION FOR SEQ ID NO:137:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 54 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:137:

TATGGATGAA GAAACTTCTC ATCAGCTGCT GTGTGATAAA TGTCCGCCGG GTAC

54

(2) INFORMATION FOR SEQ ID NO:138:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 380 amino acids  
(B) TYPE: amino acid

(ii) MOLECULE TYPE: protein

10	Glu	Thr	Leu	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp	Pro	Glu	Thr	Gly	His
	1				5						10				15	
	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Ala	Pro	Gly	Thr	Tyr	Leu	Lys	Gln	His
15				20					25					30		
	Cys	Thr	Val	Arg	Arg	Lys	Thr	Leu	Cys	Val	Pro	Cys	Pro	Asp	His	Ser
			35					40					45			
	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	Val	Tyr	Cys	Ser	Pro
20		50					55						60			
	Val	Cys	Lys	Glu	Leu	Gln	Ser	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His
	65					70					75					80
25	Asn	Arg	Val	Cys	Glu	Cys	Glu	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe
					85					90					95	
	Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Ser	Gly	Val	Val	Gln	Ala
30				100					105					110		
	Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Lys	Cys	Pro	Asp	Gly	Phe
			115					120					125			
	Phe	Ser	Gly	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	Ile	Lys	His	Thr	Asn
35		130					135						140			
	Cys	Ser	Thr	Phe	Gly	Leu	Leu	Leu	Ile	Gln	Lys	Gly	Asn	Ala	Thr	His
	145					150					155					160
40	Asp	Asn	Val	Cys	Ser	Gly	Asn	Arg	Glu	Ala	Thr	Gln	Lys	Cys	Gly	Ile
					165					170					175	
	Asp	Val	Thr	Leu	Cys	Glu	Glu	Ala	Phe	Phe	Arg	Phe	Ala	Val	Pro	Thr
45				180					185					190		
	Lys	Ile	Ile	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val	Asp	Ser	Leu	Pro	Gly
			195					200					205			
	Thr	Lys	Val	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile	Lys	Arg	Arg	His	Ser
50		210					215					220				
	Ser	Gln	Glu	Gln	Thr	Phe	Gln	Leu	Leu	Lys	Leu	Trp	Lys	His	Gln	Asn
	225					230					235					240

Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln Asp Ile Asp Leu Cys  
245 250 255

5 Glu Ser Ser Val Gln Arg His Leu Gly His Ser Asn Leu Thr Thr Glu  
260 265 270

Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly Lys Lys Ile Ser Pro  
275 280 285

10 Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys Ser Ser Glu Gln Leu  
290 295 300

15 Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr  
305 310 315 320

Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu Lys Thr Ser His Phe  
325 330 335

20 Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr Met Arg Phe Leu His  
340 345 350

Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile  
355 360 365

25 Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu  
370 375 380

(2) INFORMATION FOR SEQ ID NO:139:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 380 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:139:

Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His  
1 5 10 15

Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His  
20 25 30

50 Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr  
35 40 45



	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	Leu	Tyr	Cys	Ser	Pro	
	50						55					60					
5	Val	Cys	Lys	Glu	Leu	Gln	Tyr	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His	
	65					70					75					80	
	Asn	Arg	Val	Cys	Glu	Cys	Lys	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe	
					85					90					95		
10	Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Phe	Gly	Val	Val	Gln	Ala	
				100					105						110		
	Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg	Cys	Pro	Asp	Gly	Phe	
				115				120						125			
15	Phe	Ser	Asn	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	Arg	Lys	His	Thr	Asn	
	130						135						140				
20	Cys	Ser	Val	Phe	Gly	Leu	Leu	Leu	Thr	Gln	Lys	Gly	Asn	Ala	Thr	His	
	145					150						155				160	
	Asp	Asn	Ile	Cys	Ser	Gly	Asn	Ser	Glu	Ser	Thr	Gln	Lys	Cys	Gly	Ile	
					165					170						175	
25	Asp	Val	Thr	Leu	Cys	Glu	Glu	Ala	Phe	Phe	Arg	Phe	Ala	Val	Pro	Thr	
				180					185						190		
	Lys	Phe	Thr	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val	Asp	Asn	Leu	Pro	Gly	
			195					200					205				
30	Thr	Lys	Val	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile	Lys	Arg	Gln	His	Ser	
	210						215						220				
35	Ser	Gln	Glu	Gln	Thr	Phe	Gln	Leu	Leu	Lys	Leu	Trp	Lys	His	Gln	Asn	
	225					230					235					240	
	Lys	Ala	Gln	Asp	Ile	Val	Lys	Lys	Ile	Ile	Gln	Asp	Ile	Asp	Leu	Cys	
					245					250					255		
40	Glu	Asn	Ser	Val	Gln	Arg	His	Ile	Gly	His	Ala	Asn	Leu	Thr	Phe	Glu	
				260					265						270		
	Gln	Leu	Arg	Ser	Leu	Met	Glu	Ser	Leu	Pro	Gly	Lys	Lys	Val	Gly	Ala	
			275					280						285			
45	Glu	Asp	Ile	Glu	Lys	Thr	Ile	Lys	Ala	Cys	Lys	Pro	Ser	Asp	Gln	Ile	
	290						295					300					
50	Leu	Lys	Leu	Leu	Ser	Leu	Trp	Arg	Ile	Lys	Asn	Gly	Asp	Gln	Asp	Thr	
	305					310					315					320	
	Leu	Lys	Gly	Leu	Met	His	Ala	Leu	Lys	His	Ser	Lys	Thr	Tyr	His	Phe	
					325					330					335		

Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His  
340 345 350

5 Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile  
355 360 365

10 Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu  
370 375 380

(2) INFORMATION FOR SEQ ID NO:140:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:140:

TGGACCACCC AGAAGTACCT TCATTATGAC

30

(2) INFORMATION FOR SEQ ID NO:141:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:141:

GTCATAATGA AGGTACTTCT GGGTGGTCCA

30

(2) INFORMATION FOR SEQ ID NO:142:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:142:

GGACCACCCA GCTTCATTAT GACGAAGAAA C

31

5 (2) INFORMATION FOR SEQ ID NO:143:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:143:

20 GTTCTTCGT CATAATGAAG CTGGGTGGTC C

31

(2) INFORMATION FOR SEQ ID NO:144:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:144:

GTGGACCACC CAGGACGAAG AAACCTCTC

29

(2) INFORMATION FOR SEQ ID NO:145:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:145:

GAGAGGTTTC TTCGTCCTGG GTGGTCCAC

29

(2) INFORMATION FOR SEQ ID NO:146:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:146:

CGTTTCCTCC AAAGTTCCTT CATTATGAC

29

(2) INFORMATION FOR SEQ ID NO:147:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:147:

GTCATAATGA AGGAACTTTG GAGGAAACG

29

(2) INFORMATION FOR SEQ ID NO:148:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:148:

GGAAACGTTT CCTGCAAAGT ACCTTCATTA TG

32

(2) INFORMATION FOR SEQ ID NO:149:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:149:

CATAATGAAG GTACTTTGCA GGAAACGTTT CC

32

15

(2) INFORMATION FOR SEQ ID NO:150:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:150:

CACGCAAAAG TCGGGAATAG ATGTCAC

27

30

(2) INFORMATION FOR SEQ ID NO:151:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:151:

45

GTGACATCTA TTCCCGACTT TTGCGTG

27

(2) INFORMATION FOR SEQ ID NO:152:

50

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:152:

10 CACCCTGTCG GAAGAGGCCT TCTTC

25

(2) INFORMATION FOR SEQ ID NO:153:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:153:

25 GAAGAAGGCC TCTTCCGACA GGGTG

25

(2) INFORMATION FOR SEQ ID NO:154:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:154:

TGACCTCTCG GAAAGCAGCG TGCA

24

(2) INFORMATION FOR SEQ ID NO:155:

45

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:155:

5

TGCACGCTGC TTTCCGAGAG GTCA

24

(2) INFORMATION FOR SEQ ID NO:156:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:156:

CCTCGAAATC GAGCGAGCAG CTCC

24

(2) INFORMATION FOR SEQ ID NO:157:

25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:157:

CGATTTCGAG GTCTTTCTCG TTCTC

25

40

(2) INFORMATION FOR SEQ ID NO:158:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:158:

CCGTGAAAAT AAGCTCGTTA TAACTAGGAA TGG

33

5 (2) INFORMATION FOR SEQ ID NO:159:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:159:

CCATTCCTAG TTATAACGAG CTTATTTTCA CGG

33

(2) INFORMATION FOR SEQ ID NO:160:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:160:

CCTCTGAGCT CAAGCTTCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:161:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs

(B) TYPE: nucleic acid

40 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:161:

CCTCTCTCGA GTCAGGTGAC ATCTATTCCA CACTTTTGCG TGGC

44



(2) INFORMATION FOR SEQ ID NO:162:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:162:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:163:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:163:

CCTCTCTCGA GTCAAGGAAC AGCAAACCTG AAGAAGGC

38

(2) INFORMATION FOR SEQ ID NO:164:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:164:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:165:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 38 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:165:

CCTCTCTCGA GTCACTCTGT GGTGAGGTTT GAGTGGCC

38

(2) INFORMATION FOR SEQ ID NO:166:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 38 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:166:

CCTCTGAGCT CAAGCTTCCG AGGACCACAA TGAACAAG

38

(2) INFORMATION FOR SEQ ID NO:167:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 38 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:167:

CCTCTCTCGA GTCAGGATGT TTTCAAGTGC TTGAGGGC

38

(2) INFORMATION FOR SEQ ID NO:168:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 16 amino acids  
    (B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:168:

Met Lys His His His His His His His Ala Ser Val Asn Ala Leu Glu

10

1

5

10

15

Met Lys His His His His His His His Ala Ser Val Asn Ala Leu Glu

WHAT IS CLAIMED IS:

1. An isolated nucleic acid encoding a  
5 polypeptide comprising at least one of the biological  
activities of OPG wherein the nucleic acid is selected  
from the group consisting of:
  - a) the nucleic acids shown in Figures  
2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D  
10 (SEQ ID NO:124) or complementary strands thereof;
  - b) nucleic acids which hybridize under  
stringent conditions with the polypeptide-encoding  
regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-  
9B (SEQ ID NO:122) and 9C-9D (SEQ ID NO:124);
  - 15 c) nucleic acids which hybridize under  
stringent conditions with nucleotides 148 through 337  
inclusive as shown in Figure 1A; and
  - d) nucleic acid which are degenerate to  
the nucleic acids of (a), (b) and (c).
- 20 2. The nucleic acid of Claim 1 which is  
cDNA, genomic DNA, synthetic DNA or RNA.
3. A polypeptide encoded by the nucleic acid  
25 of Claim 1.
4. The nucleic acid of Claim 1 including one  
or more codons preferred for Escherichia coli  
expression.
- 30 5. The nucleic acid of Claim 1 having a  
detectable label attached thereto.
6. The nucleic acid of Claim 1 comprising  
35 the polypeptide-encoding region of Figure 2B-2C (SEQ ID

NO:120), Figure 9A-9B (SEQ ID NO:122) or Figure 9C-9D (SEQ ID NO:124).

7. The nucleic acid of Claim 6 having the  
5 sequence as shown in Figure 9C-D (SEQ ID NO:124) from nucleotides 158-1297.

8. An expression vector comprising the  
nucleic acid of Claim 1.

10

9. The expression vector of Claim 8 wherein  
the nucleic acid comprises the polypeptide - encoding  
region as shown in Figure 9C-9D (SEQ ID NO:124).

15

10. A host cell transformed or transfected  
with the expression vector of Claim 8.

11. The host cell of Claim 10 which is a  
eucaryotic cell.

20

12. The host cell of Claim 11 which is  
selected from the group consisting of CHO, COS, 293,  
3T3, CV-1 and BHK cells.

25

13. The host cell of Claim 10 which is a  
procaryotic cell.

14. The host cell of Claim 13 which is  
Escherichia coli.

30

15. A transgenic mammal comprising the  
expression vector of Claim 8.

16. The transgenic mammal of Claim 15 which  
35 is a rodent.

17. The transgenic mammal of Claim 16 which is a mouse.

18. A process for the production of OPG  
5 comprising:  
growing under suitable nutrient  
conditions host cells transformed or transfected with  
the nucleic acid of Claim 1; and  
isolating the polypeptide products of  
10 the expression of the nucleic acids.

19. A purified and isolated polypeptide comprising OPG.

20. The polypeptide of Claim 19 which is mammalian OPG.

21. The polypeptide of Claim 20 which is human OPG.

22. The polypeptide of Claim 19 which is substantially free of other human proteins.

23. The polypeptide of Claim 21 having the  
25 amino acid sequence as shown in Figure 2B-2C (SEQ ID NO:121), Figure 9A-9B (SEQ ID NO:123), or Figure 9C-9D (SEQ ID NO:125) or a derivative thereof.

24. The polypeptide of Claim 23 having the  
30 amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 22-401 inclusive.

25. The polypeptide of Claim 23 having the  
amino acid sequence as shown in Figure 9C-9D (SEQ ID  
35 NO:125) from residues 32-401 inclusive.

26. The polypeptide of Claim 19 which is characterized by being a product of expression of an exogenous DNA sequence.

5           27. The polypeptide of Claim 26 wherein the DNA is cDNA, genomic DNA or synthetic DNA.

28. The polypeptide of Claim 19 which has been modified with a water-soluble polymer.

10

29. The polypeptide of Claim 28 wherein the water soluble polymer is polyethylene glycol.

30. A polypeptide comprising:  
15           an amino acid sequence of at least about 164 amino acids comprising four cysteine-rich domains characteristic of the cysteine rich domains of tumor necrosis factor receptor extracellular regions; and  
            an activity of increasing bone density.

20

31. A polypeptide comprising the amino acid sequence as shown in Figure 2B-2C (SEQ ID NO:121), Figure 9A-9B (SEQ ID NO:123) or Figure 9C-9D (SEQ ID NO:125) having an amino terminus at residue 22, and  
25           wherein from 1 to 216 amino acids are deleted from the carboxy terminus.

32. The polypeptide of Claim 31 comprising the amino acid sequence from residues 22-185, 22-189,  
30           22-194, or 22-201 inclusive.

33. The polypeptide of Claim 32 further comprising an Fc region of human IgG1 extending from the carboxy terminus.

35

34. A polypeptide comprising the amino acid sequence as shown in Figure 2B-2C (SEQ ID NO:121), Figure 9A-9B (SEQ ID NO:123) or Figure 9C-9D (SEQ ID NO:125) having an amino terminus at residue 22, wherein  
5 from 1 to 10 amino acids are deleted from the amino terminus and, optionally, from 1 to 216 amino acids are deleted from the carboxy terminus.

35. The polypeptide of Claim 34 comprising  
10 the amino acid sequence from residues 27-185, 27-189, 27-194, 27-401, or 32-401 inclusive.

36. The polypeptide of Claim 35 further comprising an Fc region of human IgG1 extending from  
15 the carboxy terminus.

37. A polypeptide selected from the group consisting of:

20 huOPG [22-201]-Fc  
huOPG [22-401]-Fc  
huOPG [22-180]-Fc  
huOPG met [22-401]-Fc  
huOPG Fc-met [22-401]  
huOPG met [22-185]  
25 huOPG met [22-189]  
huOPG met [22-194]  
huOPG met [27-185]  
huOPG met [27-189]  
huOPG met [27-194]  
30 huOPG met [32-401]  
huOPG met-lys[22-401]  
huOPG met [22-401]  
huOPG met [22-401]-Fc (P25A)  
huOPG met [22-401] (P25A)  
35 huOPG met [22-401] (P26A)  
huOPG met [22-401] (P26D)



huOPG met [22-194] (P25A)  
huOPG met [22-194] (P26A)  
huOPG met met-(lys)<sub>3</sub> [22-401]  
huOPG met met-arg-gly-ser-(his)<sub>6</sub> [22-401]

5

38. A nucleic acid encoding the polypeptide  
of Claim 37.

39. An antibody or fragment thereof which  
10 specifically binds to OPG.

40. The antibody of Claim 39 which is a  
monoclonal antibody.

15 41. A method for detecting the presence of  
OPG in a biological sample comprising:

incubating the sample with the antibody of  
Claim 39 under conditions that allow binding of the  
antibody to OPG; and

20 detecting the bound antibody.

42. A method to assess the ability of a candidate substance to bind to OPG comprising:  
incubating OPG with the candidate substance  
5 under conditions that allow binding; and  
measuring the bound substance.

43. A method of regulating the levels of OPG in an animal comprising modifying the animal with a  
10 nucleic acid encoding OPG.

44. The method of Claim 43 wherein the nucleic acid promotes an increase in the tissue level of OPG.  
15

45. The method of Claim 44 wherein the animal is a human.

46. A pharmaceutical composition comprising  
20 a therapeutically effective amount of OPG in a pharmaceutically acceptable carrier, adjuvant, solubilizer, stabilizer and/or anti-oxidant.

47. The composition of Claim 46 wherein the  
25 OPG is human OPG.

48. The composition of Claim 47 wherein the OPG has the amino acid sequence as shown in Figure 9B.

49. A method of treating a bone disorder comprising administering a therapeutically effective amount of the polypeptide of Claim 19.  
30

50. The method of Claim 49 wherein the  
35 polypeptide is human OPG.

51. The method of Claim 49 wherein the bone disorder is excessive bone loss.

52. The method of Claim 51 wherein the bone disorder is selected from the group consisting of osteoporosis, Paget's disease of bone, hypercalcemia, hyperparathyroidism, steroid-induced osteopenia, bone loss due to rheumatoid arthritis, bone loss due to osteomyelitis, osteolytic metastasis, and periodontal bone loss.

53. The method of Claim 49 further comprising administering a therapeutically effective amount of a substances selected from the group consisting of bone morphogenic proteins BMP-1 through BMP-12, TGF- $\beta$  family members, IL-1 inhibitors, TNF $\alpha$  inhibitors, parathyroid hormone and analogs thereof, parathyroid hormone related protein and analogs thereof, E series prostaglandins, bisphosphonates, and bone-enhancing minerals.

54. An osteoprotegerin multimer consisting of osteoprotegerin monomers.

55. The multimer of Claim 54 which is a dimer.

56. The multimer of Claim 54 formed by interchain disulfide bonds.

57. The multimer of Claim 54 formed by association Fc regions derived from human IgG1.

58. The multimer of Claim 54 which is essentially free of osteoprotegerin monomers and inactive multimers.

59. The multimer of Claim 54 wherein the monomers comprise the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 22-401, or a derivative thereof.

60. The multimer of Claim 54 wherein the monomers comprise the amino acid sequence as shown in Figure 9C-9D (SEQ ID NO:125) from residues 22-194.

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ABSTRACT OF THE INVENTION

The present invention discloses a novel  
secreted polypeptide, termed osteoprotegerin, which is  
5 a member of the tumor necrosis factor receptor  
superfamily and is involved in the regulation of bone  
metabolism. Also disclosed are nucleic acids encoding  
osteoprotegerin, polypeptides, recombinant vectors and  
host cells for expression, antibodies which bind OPG,  
10 and pharmaceutical compositions. The polypeptides are  
used to treat bone diseases characterized by increased  
resorption such as osteoporosis.

SECRETED

FIG.1A

```

148      178      208      238      268      298
FRI-1  ALLVFLDIIEWTTQETFFPKYLHYDPETGRQLLCDKCAPGTYLKQHCTVRRKTL CVPCPD
SW: TNR2_HUMAN  HALPAQVAFTFYAPEPGSTCRLREYYDQTAQMCCSKCSPGQHAKVFCTKTSDTVCDSCED
                30      40      50      60      70      80
                |: | | | | | | | | | | | | | | | | | | | | | | | | | | | |
328
FRI-1  YSYTDSWHTS
      : | | : | :
SW: TNR2_HUMAN  STYTQLWNWVPECLSCGSRSSDQVETQACTREQNRICTRPGWYCALSKQEGCRLCAPL
                90      100     110     120     130     140

```

FIG.1B

```

FRI-1  69 YLHYDPETGRQLLCDKCAPGTYLKQHC.TVRRKTL CV.PCPDY.SYTD SW
      | | . . . | . | | | : | . | : | | | | : | . | .
TNFR profile  6 YHYDQNGRMCEECHMCQPGHFLVKHCKQPKRDTVCHKPCPEPGVTYTD DW
      116 H
      |
TNFR profile  56 H
                Z Score = 8.29

```

FIG. 1C

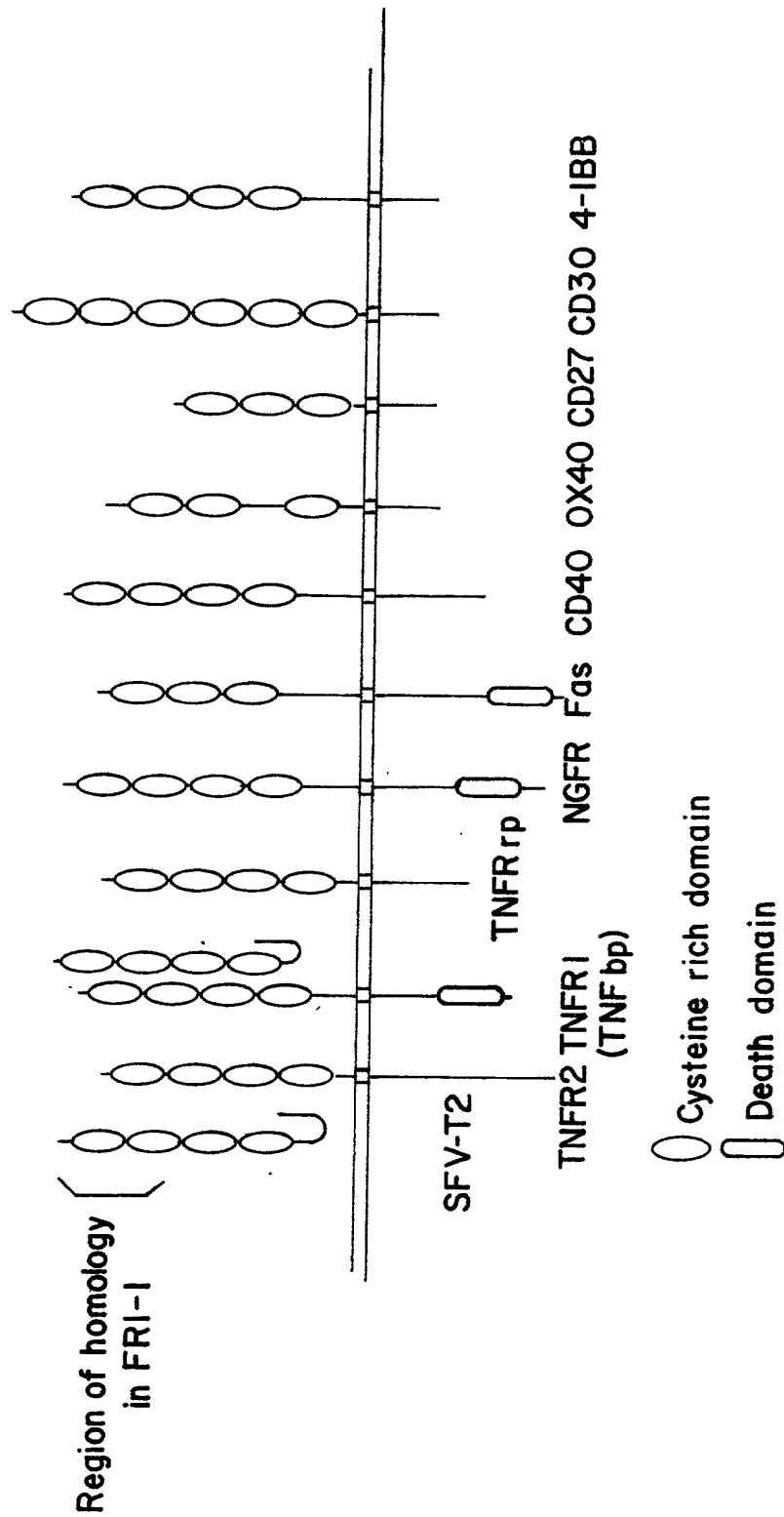


FIG.2A

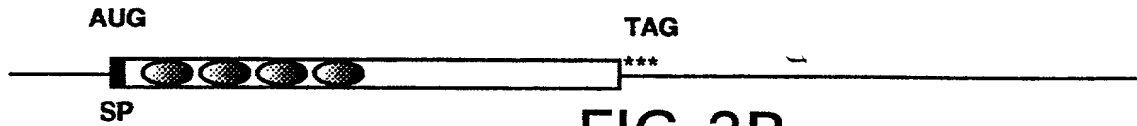


FIG.2B

10 30 50  
 ATCAAAGGCAGGGCATACTTCCTGTTGCCAGACCTTATATAAAACGTCATGTTGCCTG  
 70 90 110  
 GGCAGCAGAGAAGCACCTAGCACTGGCCAGCGGCTGCCGCTGAGGTTTCCAGAGGACC  
 130 150 170  
 ACAATGAACAAGTGGCTGTGCTGTGCACTCCTGGTGTCTTGGACATCATTTGAATGGACA  
 M N K W L C C A L L V F L D I I E W T  
 190 210 230  
 ACCCAGGAAACCTTTCCTCCAAAATACTTGCATTATGACCCAGAAACCGGACGTCAGCTC  
 T O E T F P P K Y L H Y D P E T G R Q L  
 250 270 290  
 TTGTGTGACAAATGTGCTCCTGGCACCTACCTAAAACAGCACTGCACAGTCAGGAGGAAG  
 L C D K C A P G T Y L K Q H C T V R R K  
 310 330 350  
 ACACTGTGTGTCCCTTGCCCTGACTACTCTTATACAGACAGCTGGCACACGAGTGATGAA  
 T L C V P C P D Y S Y T D S W H T S D E  
 370 390 410  
 TGCCTGTACTGCAGCCCCGTGTGCAAGGAAGTGCAGACCGTGAAACAGGAGTGCAACCGC  
 C V Y C S P V C K E L Q T V K Q E C N R  
 430 450 470  
 ACCCACAACCGAGTGTGCGAATGTGAGGAAGGGCGCTACCTGGAGCTCGAATTCTGCTTG  
 T H N R V C E C E E G R Y L E L E F C L  
 490 510 530  
 AAGCACCGGAGCTGTCCCCCAGGCTTGGGTGTGCTGCAGGCTGGGACCCAGAGCGAAAC  
 K H R S C P P G L G V L Q A G T P E R N  
 550 570 590  
 ACGGTTTGCAAAAGATGTCCGGATGGGTTCTTCTCAGGTGAGACGTCATCGAAAGCACCC  
 T V C K R C P D G F F S G E T S S K A P  
 610 630 650  
 TGTAGGAAACACACCAACTGCAGCTCACTTGGCCTCCTGCTAATTCAGAAAGGAAATGCA  
 C R K H T N C S S L G L L L I Q K G N A  
 670 690 710  
 ACACATGACAATGTATGTTCCGGAACAGAGAAGCAACTCAAAATTGTGGAATAGATGTC  
 T H D N V C S G N R E A T Q N C G I D V  
 730 750 770  
 ACCCTGTGCGAAGAGGCATTCTTCAGGTTTGCTGTGCCTACCAAGATTATACCGAATTGG  
 T L C E E A F F R F A V P T K I I P N W  
 790 810 830  
 CTGAGTGTCTGTTGGTGGACAGTTTGCCTGGGACCAAAGTGAATGCAGAGAGTGTAGAGAGG  
 L S V L V D S L P G T K V N A E S V E R  
 850 870 890  
 ATAAACGGAGACACAGCTCGCAAGAGCAAACCTTCCAGCTACTTAAGCTGTGGAAGCAT  
 I K R R H S S Q E Q T F Q L L K L W K H  
 910 930 950  
 CAAAACAGAGACCAGGAAATGGTGAAGAAGATCATCCAAGACATTGACCTCTGTGAAAGC  
 Q N R D Q E M V K K I I Q D I D L C E S  
 970 990 1010  
 AGTGTGCAACGGCATATCGGCCACGCGAACCTCACCACAGAGCAGCTCCGCATCTTGATG  
 S V Q R H I G H A N L T T E Q L R I L M



# FIG.2C

```

1030      1050      1070
GAGAGCTTGCCTGGGAAGAAGATCAGCCCAGACGAGATTGAGAGAACGAGAAAGACCTGC
E  S  L  P  G  K  K  I  S  P  D  E  I  E  R  T  R  K  T  C
1090      1110      1130
AAACCCAGCGAGCAGCTCCTGAAGCTACTGAGCTTGTGGAGGATCAAAAATGGAGACCAA
K  P  S  E  Q  L  L  K  L  L  S  L  W  R  I  K  N  G  D  Q
1150      1170      1190
GACACCTTGAAGGGCCTGATGTACGCACTCAAGCACTTGAAAGCATACCACTTTCCCAA
D  T  L  K  G  L  M  Y  A  L  K  H  L  K  A  Y  H  F  P  K
1210      1230      1250
ACCGTCACCCACAGTCTGAGGAAGACCATCAGGTTCTTGCACAGCTTCACCATGTACCGA
T  V  T  H  S  L  R  K  T  I  R  F  L  H  S  F  T  M  Y  R
1270      1290      1310
TTGTATCAGAACTCTTTCTAGAAATGATAGGGAATCAGGTTCAATCAGTGAAGATAAGC
L  Y  Q  K  L  F  L  E  M  I  G  N  Q  V  Q  S  V  K  I  S
1330      1350      1370
TGCTTATAGTTAGGAATGGTCACTGGGCTGTTTCTTCAGGATGGGCCAACACTGATGGAG
C  L
1390      1410      1430
CAGATGGCTGCTTCTCCGGCTCTTGAAATGGCAGTTGATTCCTTTCTCATCAGTTGGTGG
1450      1470      1490
GAATGAAGATCCTCCAGCCCAACACACACACTGGGGAGTCTGAGTCAGGAGAGTGAGGCA
1510      1530      1550
GGCTATTTGATAATTGTGCAAAGCTGCCAGGTGTACACCTAGAAAGTCAAGCACCTGAG
1570      1590      1610
AAAGAGGATATTTTTTATAACCTCAAACATAGGCCCTTTCCTTCCTCTCCTTATGGATGAG
1630      1650      1670
TACTCAGAAGGCTTCTACTATCTTCTGTGTCATCCCTAGATGAAGGCCTCTTTTATTTAT
1690      1710      1730
TTTTTTTATTCTTTTTTTTCGGAGCTGGGGACCGAACCCAGGGCCTTGCGCTTGCGAGGCAA
1750      1770      1790
GTGCTCTACCACTGAGCTAAATCTCCAACCCCTGAAGGCCTCTTTCTTTCTGCCTCTGAT
1810      1830      1850
AGTCTATGACATTCTTTTTTCTACAATTTCGTATCAGGTGCACGAGCCTTATCCCATTTGT
1870      1890      1910
AGGTTTCTAGGCAAGTTGACCGTTAGCTATTTTTCCCTCTGAAGATTTGATTTCGAGTTGC
1930      1950      1970
AGACTTGGCTAGACAAGCAGGGGTAGGTTATGGTAGTTTATTTAACAGACTGCCACCAGG
1990      2010      2030
AGTCCAGTGTTTCTTGTTCTCTGTAGTTGTACCTAAGCTGACTCCAAGTACATTTAGTA
2050      2070      2090
TGAAAAATAATCAACAAATTTTATTCCTTCTATCAACATTGGCTAGCTTTGTTTCAGGGC
2110      2130      2150
ACTAAAAGAACTACTATATGGAGAAAGAATTGATATTGCCCCCAACGTTCAACAACCCA
2170      2190      2210
ATAGTTTATCCAGCTGTCATGCCTGGTTTCAGTGTCTACTGACTATGCGCCCTCTTATTAC
2230      2250      2270
TGCATGCAGTAATTCAACTGGAAATAGTAATAATAATAATAGAAATAAAATCTAGACTCC
2290      2310      2330
ATTGGATCTCTCTGAATATGGGAATATCTAACTTAAGAAGCTTTGAGATTTTCAGTTGTGT
2350      2370      2390
TAAAGGCTTTTATTAAAAAGCTGATGCTCTTCTGTAAAGTTACTAATATATCTGTAAGA
2410      2430
CTATTACAGTATTGCTATTTATATCCATCCAG

```



**FIG. 2E**

[illegible]

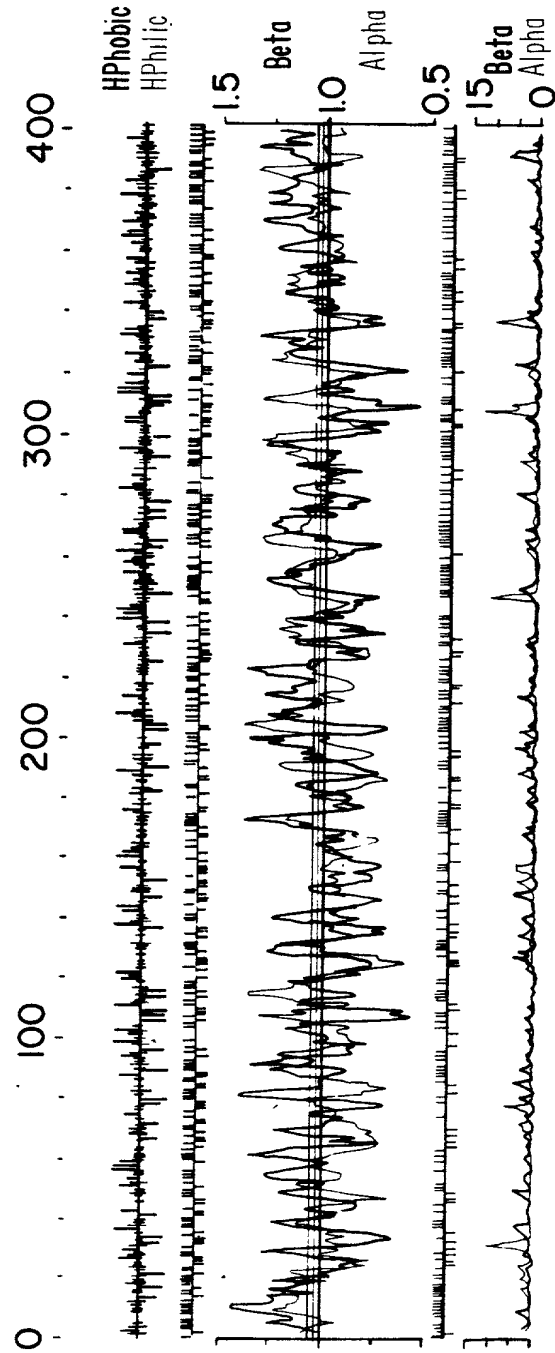


FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

FIG. 3E

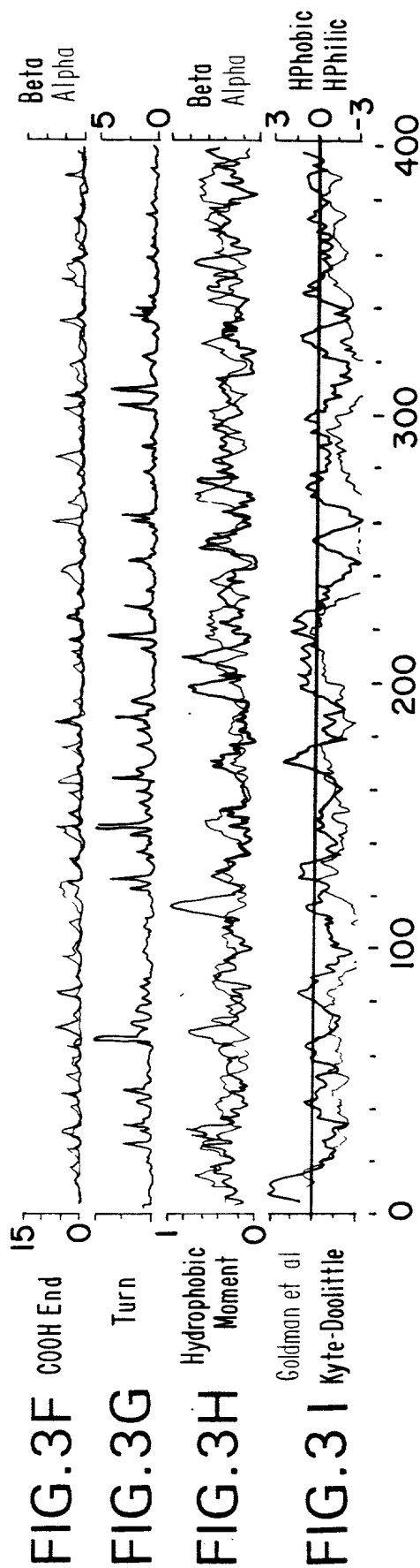


FIG.4A

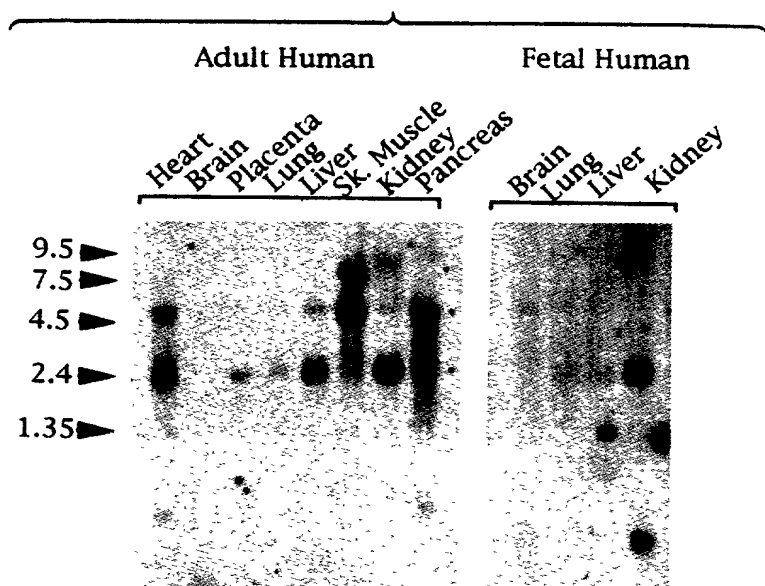


FIG.4B

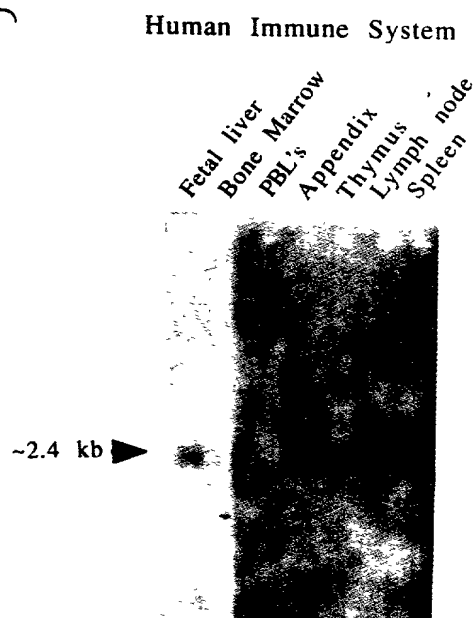
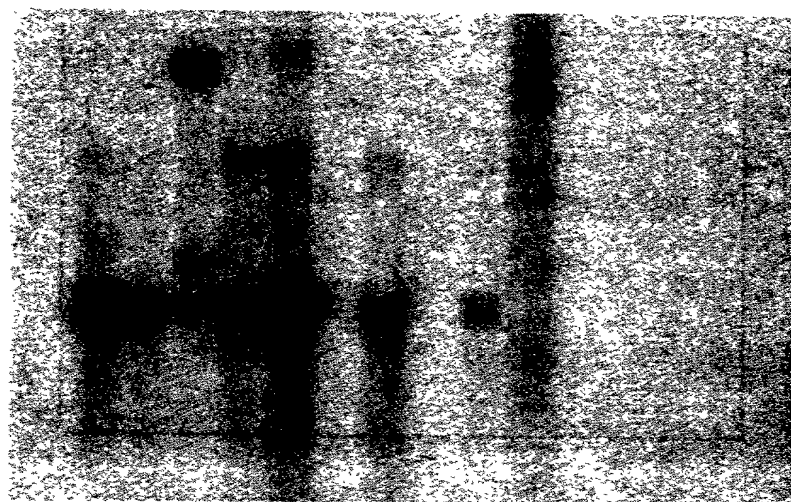


FIG.5



2 11 16 17 22 28 33 38 45 Kb 1 12 18 30  
Transgenic Founders Controls

FIG.6A

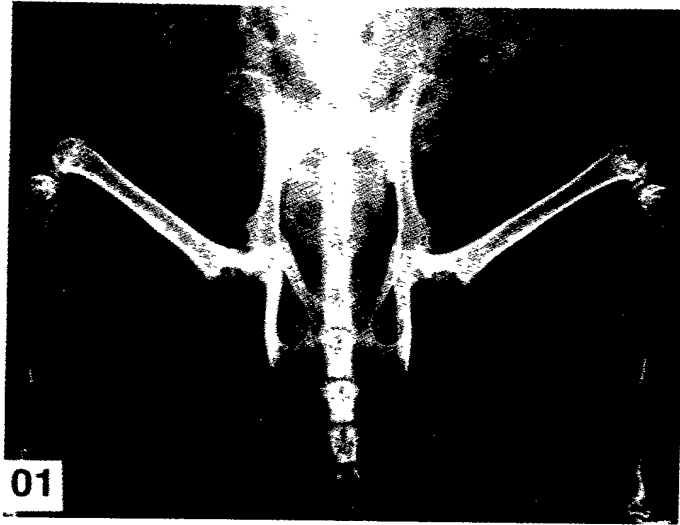


FIG.6B

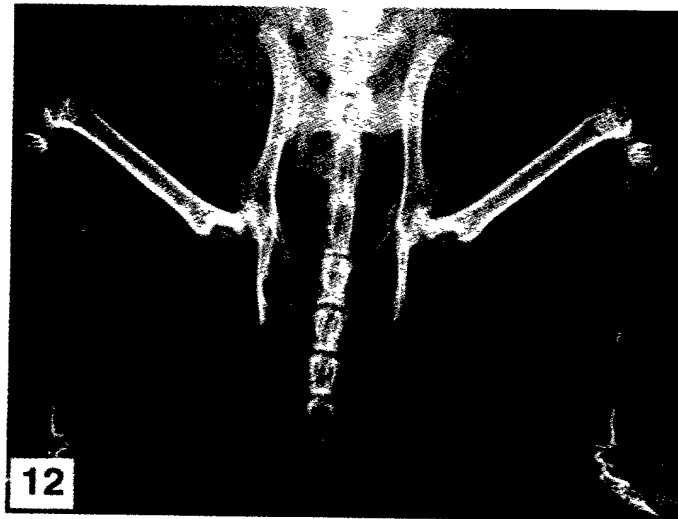


FIG.6C

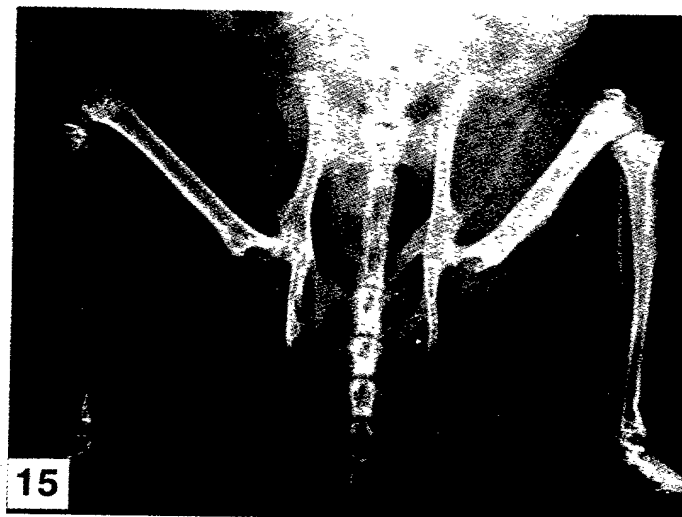




FIG.6D

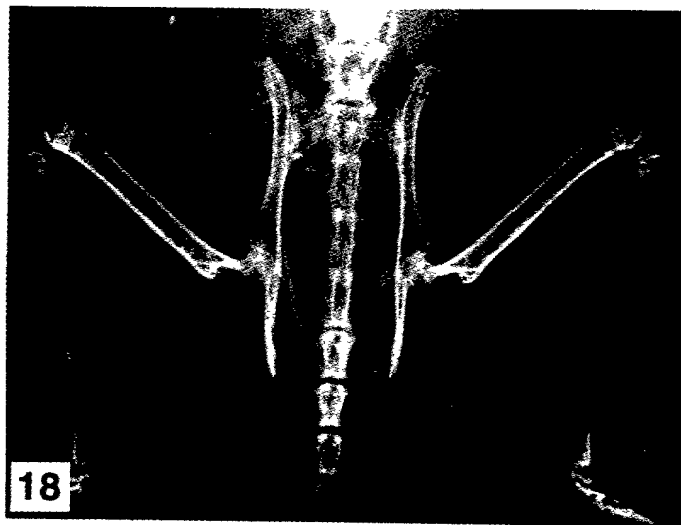


FIG.6E

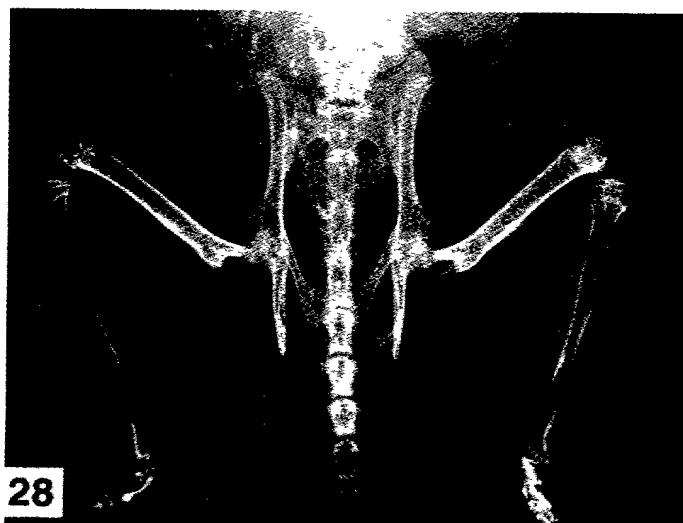


FIG.6F



FIG.6G

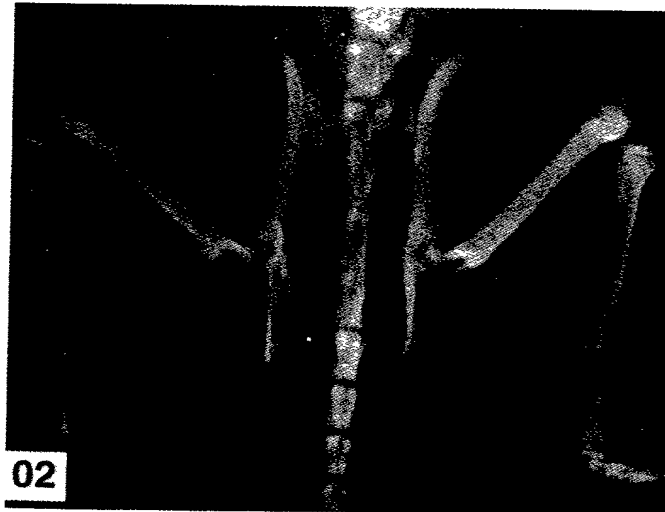


FIG.6H

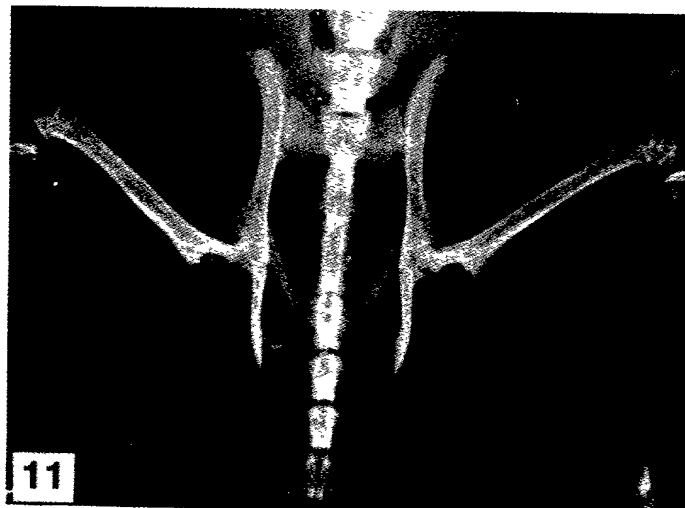


FIG.6I



FIG.6J

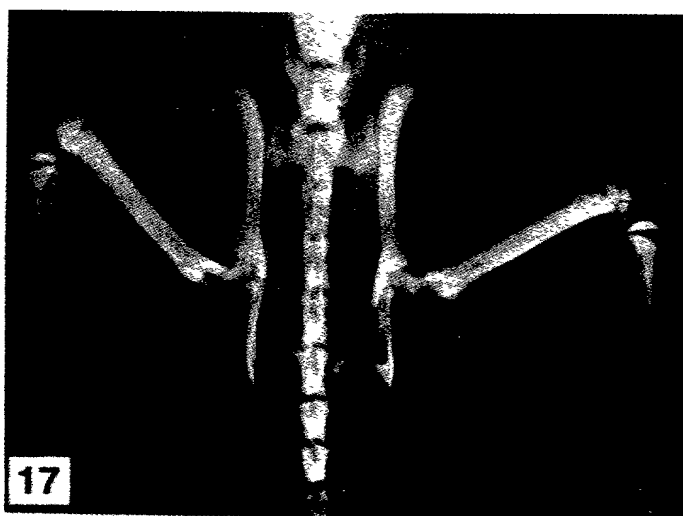


FIG.7A

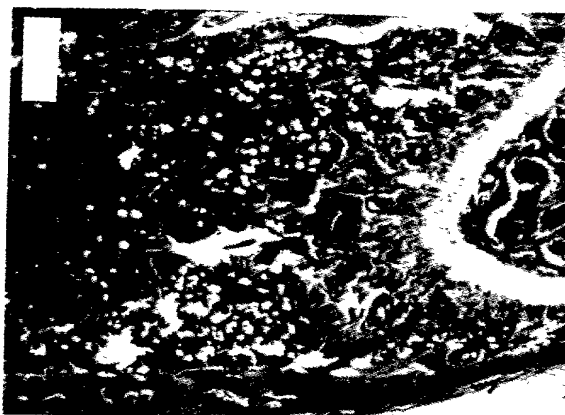


FIG.7B



FIG.7C

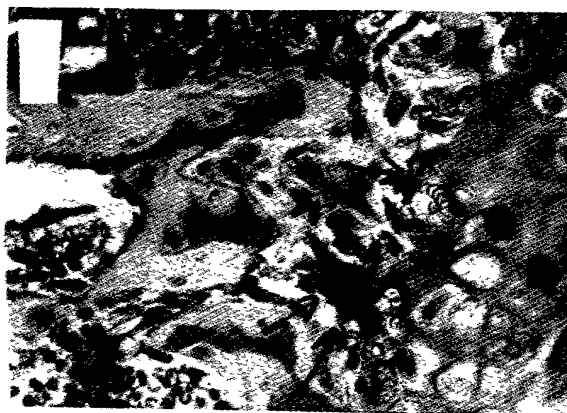


FIG.7D

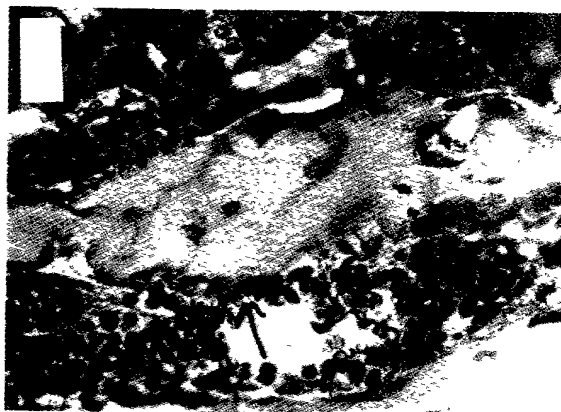


FIG.7E

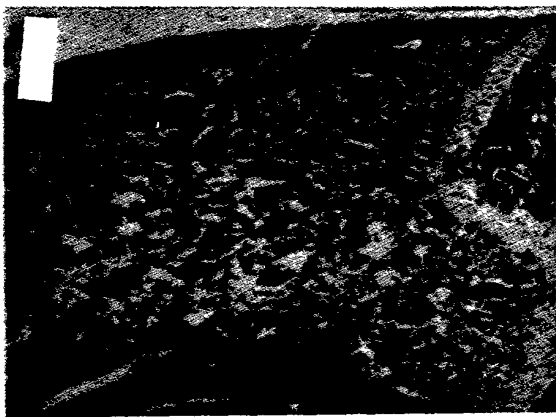


FIG.7F

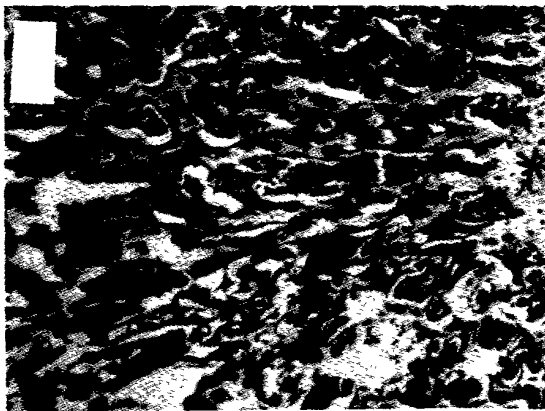


FIG.7G

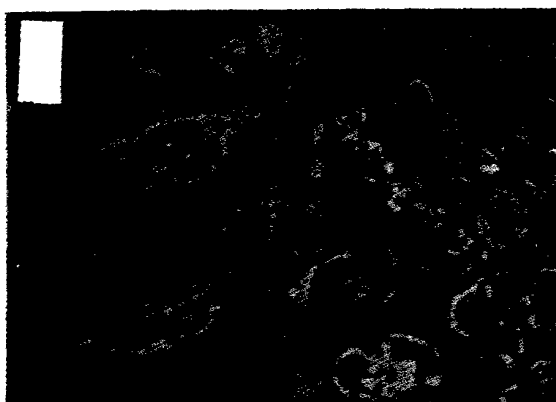


FIG.7H

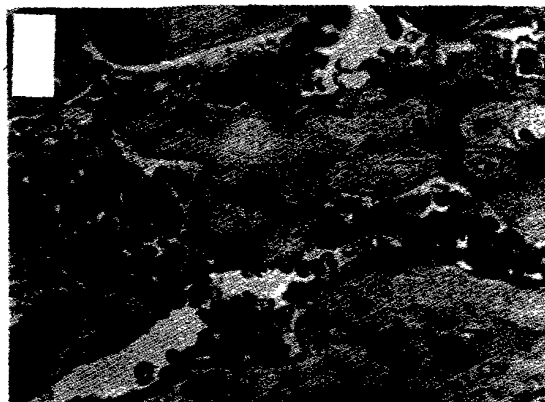


FIG.8A

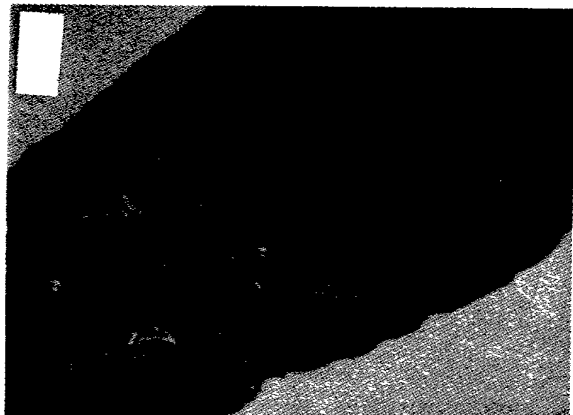


FIG.8B

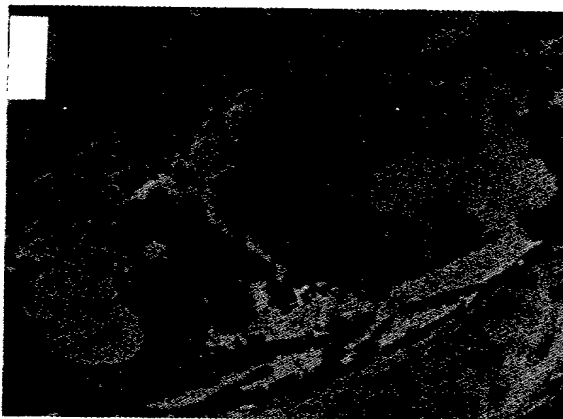
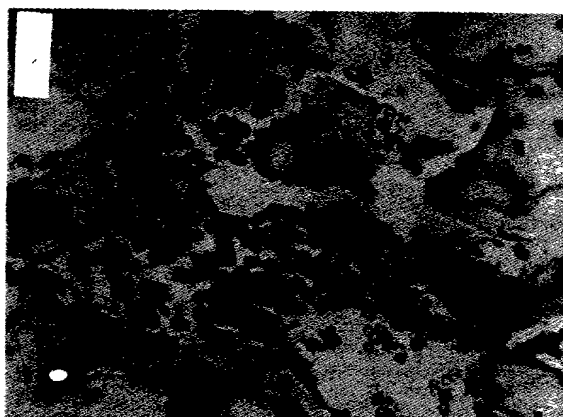


FIG.8C



FIG.8D



# FIG.9A

10 30 50  
 CCTTATATAARACGTCATGATTGCCTGGGCTGCAGAGACGCACCTAGCACTGACCCAGCG  
 70 90 110  
 GCTGCCTCCTGAGGTTTCCCGAGGACCACAATGAACAAGTGGCTGTGCTGCGCACTCCTG  
 M N K W L C C A L L  
 130 150 170  
 GTGCTCCTGGACATCATTTGAATGGACAACCCAGGAAACCCCTTCCTCCAAAGTACTTGCAT  
 V L L D I I E W T T O E T L P P K Y L H  
 190 210 230  
 TATGACCCAGAACTGGTCATCAGCTCCTGTGTGACAAATGTGCTCCTGGCACCTACCTA  
 Y D P E T G H Q L L C D K C A P G T Y L  
 250 270 290  
 AAACAGCACTGCACAGTGAGGAGGAAGACATTTGTGTGTCCCTTGCCCTGACCACTCTTAT  
 K Q H C T V R R K T L C V P C P D H S Y  
 310 330 350  
 ACGGACAGCTGGCACACCAGTGATGAGTGTGTGTATTGCAGCCCAGTGTGCAAGGAAGT  
 T D S W H T S D E C V Y C S P V C K E L  
 370 390 410  
 CAGTCCGTGAAGCAGGAGTGCAACCGCACCCACAACCGAGTGTGTGAGTGTGAGGAAGGG  
 Q S V K Q E C N R T H N R V C E C E E G  
 430 450 470  
 CGTTACCTGGAGATCGAATTCTGCTTGAAGCACCGGAGCTGTCCCCGGGCTCCGGCGTG  
 R Y L E I E F C L K H R S C P P G S G V  
 490 510 530  
 GTGCAAGCTGGAACCCAGAGCGAAACACAGTTTGCAAAAAATGTCCAGATGGGTTCTTC  
 V Q A G T P E R N T V C K K C P D G F F  
 550 570 590  
 TCAGGTGAGACTTCATCGAAAGCACCCGTGTATAAAACACACGAACTGCAGCACATTTGGC  
 S G E T S S K A P C I K H T N C S T F G  
 610 630 650  
 CTCCTGCTAATTCAGAAAGGAAATGCAACACATGACAACGTGTGTTCGGGAAACAGAGAA  
 L L L I Q K G N A T H D N V C S G N R E  
 670 690 710  
 GCCACGCAAAAGTGTGGAATAGATGTCACCCTGTGTGAAGAGGCCTTCTTCAGGTTTGCT  
 A T Q K C G I D V T L C E E A F F R F A  
 730 750 770  
 GTTCCTACCAAGATTATACCAAATTGGCTGAGTGTTTTGGTGGACAGTTTGCCTGGGACC  
 V P T K I I P N W L S V L V D S L P G T

# FIG.9B

790 810 830  
 AAAGTGAATGCCGAGAGTGTAGAGAGGATAAAACGGAGACACAGCTCACAAGAGCAAACC  
 K V N A E S V E R I K R R H S S Q E Q T  
 850 870 890  
 TTCCAGCTGCTGAAGCTGTGGAACATCAAAACAGAGACCAGGAAATGGTGAAGAAGATC  
 F Q L L K L W K H Q N R D Q E M V K K I  
 910 930 950  
 ATCCAAGACATTGACCTCTGTGAAAGCAGCGTGCAGCGGCATCTCGGCCACTCGAACCTC  
 I Q D I D L C E S S V Q R H L G H S N L  
 970 990 1010  
 ACCACAGAGCAGCTTCTTGCCTTGATGGAGAGCCTGCCTGGGAAGAAGATCAGCCCAGAA  
 T T E Q L L A L M E S L P G K K I S P E  
 1030 1050 1070  
 GAGATTGAGAGAACGAGAAAGACCTGCAAATCGAGCGAGCAGCTCCTGAAGCTACTCAGT  
 E I E R T R K T C K S S E Q L L K L L S  
 1090 1110 1130  
 TTATGGAGGATCAAAAATGGTGACCAAGACACCTTGAAGGGCCTGATGTATGCCCTCAAG  
 L W R I K N G D Q D T L K G L M Y A L K  
 1150 1170 1190  
 CACTTGAAAACATCCCACTTTCCCAAAACTGTCACCCACAGTCTGAGGAAGACCATGAGG  
 H L K T S H F P K T V T H S L R K T M R  
 1210 1230 1250  
 TTCCTGCACAGCTTCACAATGTACAGACTGTATCAGAAGCTCTTTTGTAGAAATGATAGGG  
 F L H S F T M Y R L Y Q K L F L E M I G  
 1270 1290 1310  
 AATCAGGTTCAATCCGTGAAAATAAGCTGCTTATAACTAGGAATGGTCACTGGGCTGTTT  
 N Q V Q S V K I S C L  
 CTTCA



# FIG.9C

```

      10              30              50
GTATATATAACGTGATGAGCGTACGGGTGCGGAGACGCACCGGAGCGCTCGCCCAGCCGC
      70              90              110
CGYCTCCAAGCCCCCTGAGGTTTCCGGGGACCACAATGAACAAGTTGCTGTGCTGCGCGCT
                                M N K L L C C A L
      130              150              170
CGTGT'TTCTGGACATCTCCATTAAGTGGACCACCCAGGAAACGTTTCCTCCAAAGTACCT
V F L D I S I K W T T O E T F P P K Y L
      190              210              230
TCATTATGACGAAGAAACCTCTCATCAGCTGTTGTGTGACAAATGTCTCCTGGTACCTA
H Y D E E T S H Q L L C D K C P P G T Y
      250              270              290
CCTAAAACAACACTGTACAGCAAAGTGGAAGACCGTGTGCGCCCCCTTGCCCTGACCACTA
L K Q H C T A K W K T V C A P C P D H Y
      310              330              350
CTACACAGACAGCTGGCACACCAGTGACGAGTGTCTATACTGCAGCCCCGTGTGCAAGGA
Y T D S W H T S D E C L Y C S P V C K E
      370              390              410
GCTGCAGTACGTCAAGCAGGAGTGCAATCGCACCCACAACCGCGTGTGCGAATGCAAGGA
L Q Y V K Q E C N R T H N R V C E C K E
      430              450              470
AGGGCGCTACCTTGAGATAGAGTTCTGCTTGAAACATAGGAGCTGCCCTCCTGGATTTGG
G R Y L E I E F C L K H R S C P P G F G
      490              510              530
AGTGGTGCAAGCTGGAACCCCAGAGCGAAATACAGTTTGCAAAGATGTCCAGATGGGTT
V V Q A G T P E R N T V C K R C P D G F
      550              570              590
CTTCTCAAATGAGACGTCATCTAAAGCACCCCTGTAGAAAACACACAAATTGCAGTGTCTT
F S N E T S S K A P C R K H T N C S V F
      610              630              650
TGGTCTCCTGCTAACTCAGAAAGGAAATGCAACACACGACAACATATGTTCCGGAAACAG
G L L L T Q K G N A T H D N I C S G N S
      670              690              710
TGAATCAACTCAAAAATGTGGAATAGATGTTACCCTGTGTGAGGAGGCATTCTTCAGGTT
E S T Q K C G I D V T L C E E A F F R F
      730              750              770
TGCTGTTCTTACAAAGTTTACGCCTAACTGGCTTAGTGTCTTGGTAGACAATTTGCCTGG
A V P T K F T P N W L S V L V D N L P G

```

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# FIG.9D

790 810 830  
 CACCAAAGTAAACGCAGAGAGTGTAGAGAGGATAAAACGGCAACACAGCTCACAAGAACA  
 T K V N A E S V E R I K R Q H S S Q E Q  
 850 870 890  
 GACTTTCAGCTGCTGAAGTTATGGAAACATCAAAACAAAGACCAAGATATAGTCAAGAA  
 T F Q L L K L W K H Q N K D Q D I V K K  
 910 930 950  
 GATCATCCAAGATATTGACCTCTGTGAAAACAGCGTGCAGCGGCACATTGGACATGCTAA  
 I I Q D I D L C E N S V Q R H I G H A N  
 970 990 1010  
 CCTCACCTTCGAGCAGCTTCGTAGCTTGATGGAAAGCTTACCGGGAAGAAAGTGGGAGC  
 L T F E Q L R S L M E S L P G K K V G A  
 1030 1050 1070  
 AGAAGACATTGAAAAACAATAAAGGCATGCAAACCCAGTGACCAGATCCTGAAGCTGCT  
 E D I E K T I K A C K P S D Q I L K L L  
 1090 1110 1130  
 CAGTTTGTGGCGAATAAAAAATGGCGACCAAGACACCTTGAAGGGCCTAATGCACGCACT  
 S L W R I K N G D Q D T L K G L M H A L  
 1150 1170 1190  
 AAAGCACTCAAAGACGTACCACTTTCCCAAAACTGTCACTCAGAGTCTAAAGAAGACCAT  
 K H S K T Y H F P K T V T Q S L K K T I  
 1210 1230 1250  
 CAGGTTCTTCACAGCTTCACAATGTACAAATTGTATCAGAAGTTATTTTGTAGAAATGAT  
 R F L H S F T M Y K L Y Q K L F L E M I  
 1270 1290 1310  
 AGGTAACCAGGTCCAATCAGTAAAAATAAGCTGCTTATAACTGGAAATGGCCATTGAGCT  
 G N Q V Q S V K I S C L  
 1330 1350  
 GTTTCCTCACAATTGGCGAGATCCCATGGATGATAA

FIG. 9E

muosteo.frg	MNKKWLLCCALLVLLDIIIEWTTTQETLPPPKYLYHYDPEETGHHQLLCCDKKCAPGTYL	50
ratosteo.frg	MNKKWLLCCALLVFLDIIIEWTTTQETLPPPKYLYHYDPEETGRQLLCCDKKCAPGTYL	50
huosteo.frg	MNKKLCCALLVFLDISIKWTTTQETLPPPKYLYHYDEETSHQLLCCDKKCPPGTYL	50
muosteo.frg	KQHCTVRRKTLCTVPCPDHSTYTDSSHHTSDSDECVYCSPPVCKEELQSVKQECNRT	100
ratosteo.frg	KQHCTVRRKTLCTVPCPDHYSTYTDSSHHTSDSDECVYCSPPVCKEELQTVKQECNRT	100
huosteo.frg	KQHCTAKWKTVCAPCPDHYSTYTDSSHHTSDSDECLYCSPPVCKEELQYVKQECNRT	100
muosteo.frg	HNRVCECEEGRYLEIEFCLKHRSCPPGSGVVQAGTPERNNTVCKKCPDGGFF	150
ratosteo.frg	HNRVCECEEGRYLELEFCLKHRSCPPGLGVQLQAGTPERNNTVCKRCPDGGFF	150
huosteo.frg	HNRVCECEKEGRYLEIEFCLKHRSCPPGFGVVQAGTPERNNTVCKRCPDGGFF	150
muosteo.frg	SGETSSSKAPCIIKHTNCS	200
ratosteo.frg	SGETSSSKAPCRKHTNCS	200
huosteo.frg	SNETSSSKAPCRKHTNCS	200

FIG. 9F

muosteo.frg	L	C	E	E	A	F	F	R	F	A	V	P	T	K	I	I	P	N	W	L	S	V	L	V	D	S	L	P	G	T	K	V	N	A	E	S	V	E	R	I	K	R	R	H	S	S	Q	E	Q	T
ratosteo.frg	L	C	E	E	A	F	F	R	F	A	V	P	T	K	I	I	P	N	W	L	S	V	L	V	D	S	L	P	G	T	K	V	N	A	E	S	V	E	R	I	K	R	R	H	S	S	Q	E	Q	T
huosteo.frg	L	C	E	E	A	F	F	R	F	A	V	P	T	K	I	P	N	W	L	S	V	L	V	D	N	L	P	G	T	K	V	N	A	E	S	V	E	R	I	K	R	H	S	S	Q	E	Q	T		

muosteo.frg	F	Q	L	L	K	L	W	K	H	Q	N	R	D	Q	E	M	V	K	K	I	I	Q	D	I	D	L	C	E	S	S	V	Q	R	H	L	G	H	S	N	L	T	T	E	Q	L	L	A	L	M	E	
ratosteo.frg	F	Q	L	L	K	L	W	K	H	Q	N	R	D	Q	E	M	V	K	K	I	I	Q	D	I	D	L	C	E	S	S	V	Q	R	H	I	G	H	A	N	L	T	T	E	Q	L	R	I	L	M	E	
huosteo.frg	F	Q	L	L	K	L	W	K	H	Q	N	K	D	Q	D	I	V	K	K	I	I	Q	D	I	D	L	C	E	N	S	V	Q	R	H	I	G	H	A	N	L	T	F	E	E	Q	L	R	S	L	M	E

muosteo.frg	S	L	P	G	K	K	I	S	P	E	E	I	E	R	T	R	K	T	C	K	S	S	E	Q	L	L	K	L	S	L	W	R	I	K	N	G	D	Q	D	T	L	K	G	L	M	Y	A	L	K	350
ratosteo.frg	S	L	P	G	K	K	I	S	P	D	E	I	E	R	T	R	K	T	C	K	P	S	E	Q	L	L	K	L	S	L	W	R	I	K	N	G	D	Q	D	T	L	K	G	L	M	Y	A	L	K	350
huosteo.frg	S	L	P	G	K	K	V	G	A	E	D	I	E	K	T	I	K	A	C	K	P	S	D	Q	I	L	K	L	S	L	W	R	I	K	N	G	D	Q	D	T	L	K	G	L	M	H	A	L	K	350

muosteo.frg	H L K T S	H F F P K T V T H S L R K T	M R F F L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C	400
ratosteo.frg	H L K A Y	H F F P K T V T H S L R K T	I R F F L H S F T M Y R L Y Q K L F L E M I G N Q V Q S V K I S C	400
huosteo.frg	H S K T Y	H F F P K T V T Q S L K K T	I R F F L H S F T M Y K L L Y Q K L F L E M I G N Q V Q S V K I S C	400

```

muosteo.frg L
ratosteo.frg L
huosteo.frg L

```

FIG.10

ltnrr	C	P	Q	-	G	K	Y	I	H	P	Q	N	N	S	I	C	C	T	K	C	H	K	G	T	Y	L	Y	N	D	C	P	G	P	G	Q	D	T	D	C	R	E	C	E	S	G	S	F	T	A	S	49
humoste	P	P	K	Y	L	H	Y	D	E	E	T	S	H	Q	L	L	C	D	K	C	P	P	G	T	Y	L	K	Q	H	C	T	A	K	-	W	K	T	V	C	A	P	C	P	D	H	Y	T	D	S	49	
ltnrr	E	N	H	L	R	H	C	L	S	C	S	-	K	C	R	K	E	M	G	Q	V	E	I	S	S	C	T	V	D	R	D	T	V	C	G	C	R	K	N	Q	Y	R	H	Y	W	S	E	N	L	F	98
humoste	W	H	T	S	D	E	C	L	Y	C	S	P	V	C	-	K	E	L	Q	Y	V	K	-	Q	E	C	N	R	T	H	N	R	V	C	E	C	K	E	G	R	Y	L	E	I	-	-	E	-	F	93	
ltnrr	Q	C	F	N	C	S	L	C	L	N	G	-	T	V	H	L	S	C	Q	E	K	Q	N	T	V	C	T	-	C	H	A	G	F	F	L	R	E	-	-	-	N	E	C	V	S	C	139				
humoste	-	C	L	K	H	R	S	C	P	P	G	F	G	V	V	Q	A	G	T	P	E	R	N	T	V	C	K	R	C	P	D	G	F	F	S	N	E	T	S	S	K	A	P	C	R	K	H	139			

FIG. II

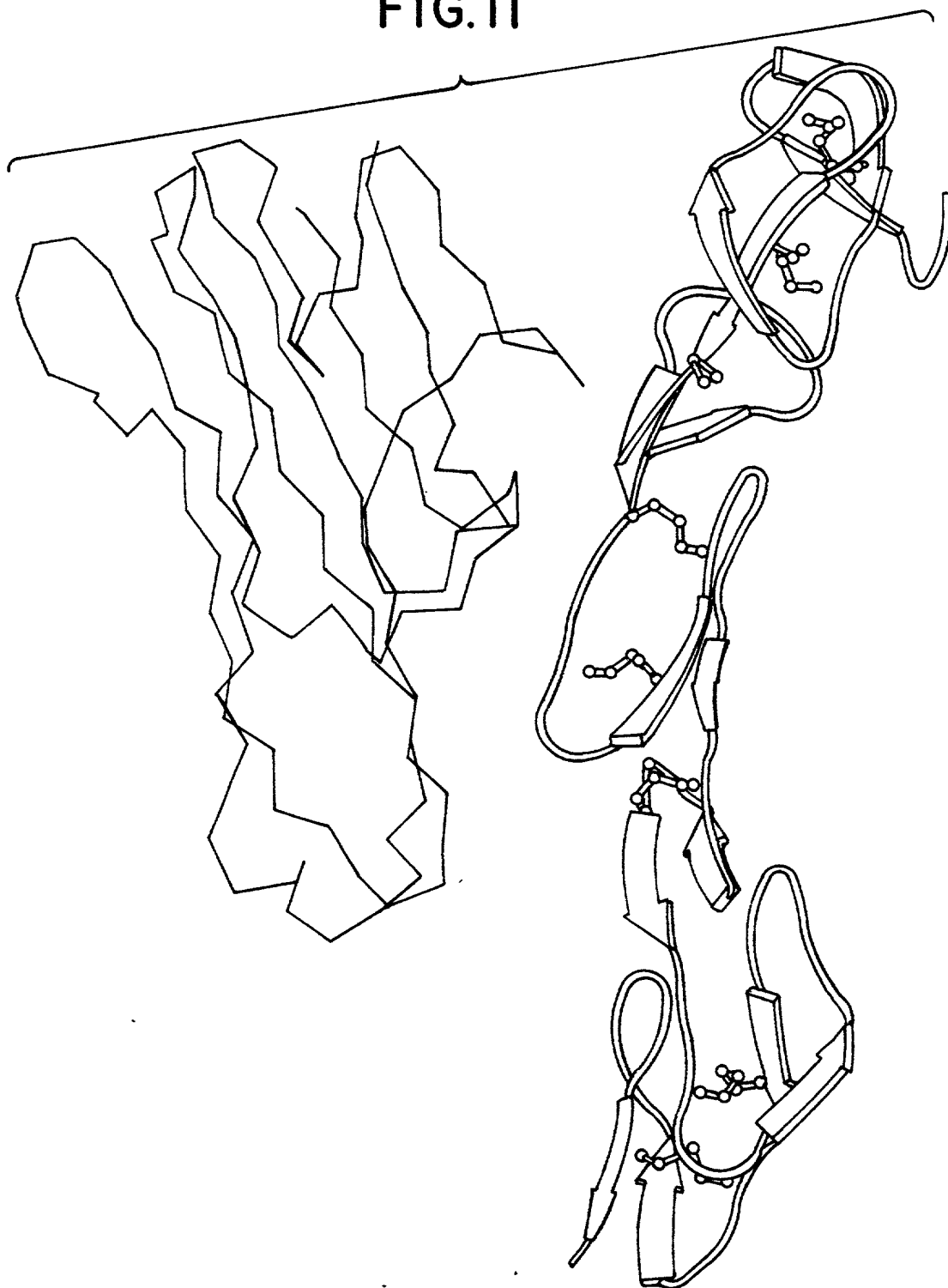


FIG.12A

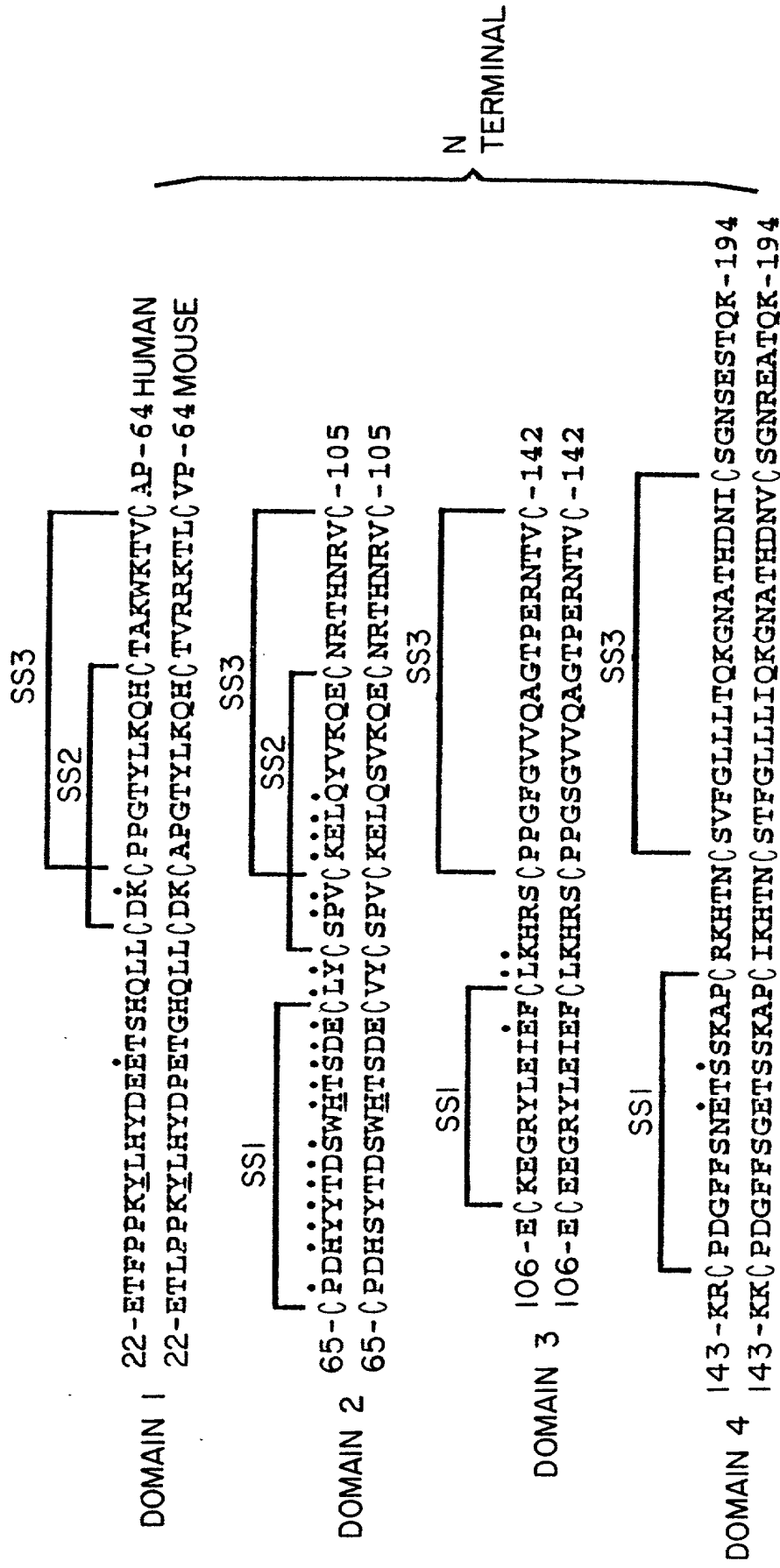


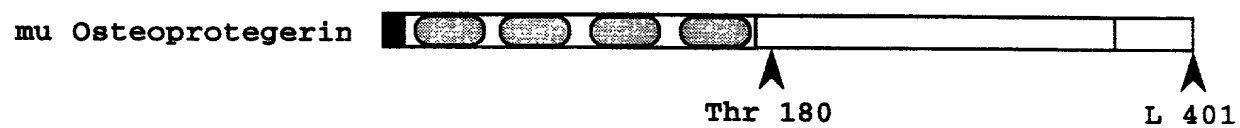
FIG.12B

195 -CGIDVTICEEAFFRFAVPTKFTPNWLSVLVDNLPGTKVNAESVERIKRQHSS-246  
 195 -CGIDVTICEEAFFRFAVPTKIIIPNWLSVLVDSLPGTKVNAESVERIKRRHSS-246  
 247 -QEQTFOQLLKLWKHKQNKDQDIVKKIIQDIDICENSVQRHGHANLTFEQLRSL-298  
 247 -QEQTFOQLLKLWKHKQNRDQEMVKKIIQDIDICESSVQRHLGHSNLTTEQLLAL-298  
 299 -MESLPGKKVGAEDIEKTIKCKPQSDQILKLLSLWRIKNGDQDTLKGMLHALK-350  
 299 -MESLPGKKISPPEIERTRKTKCSSEQLLKLLSLWRIKNGDQDTLKGMLYALK-350  
 351 -HSKTYHFPKTVTQSLKKTIRFLHSFTMYKLYQKLFLEMIGNQVQSVKISCL-401  
 351 -HLKTSHPKTVVTHSLRKTMRFLLHSFTMYRLYQKLFLEMIGNQVQSVKISCL-401

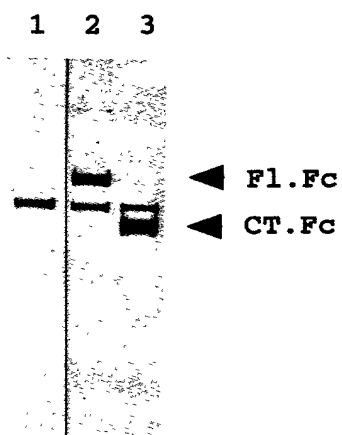
C } TERMINAL



# FIG.13A



# FIG.13B



# FIG.13C

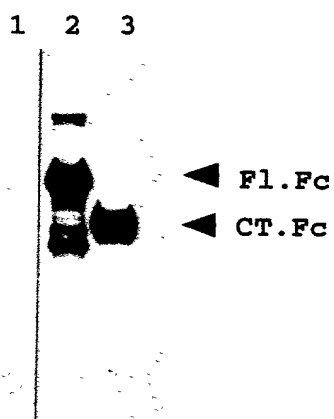


FIG.14A

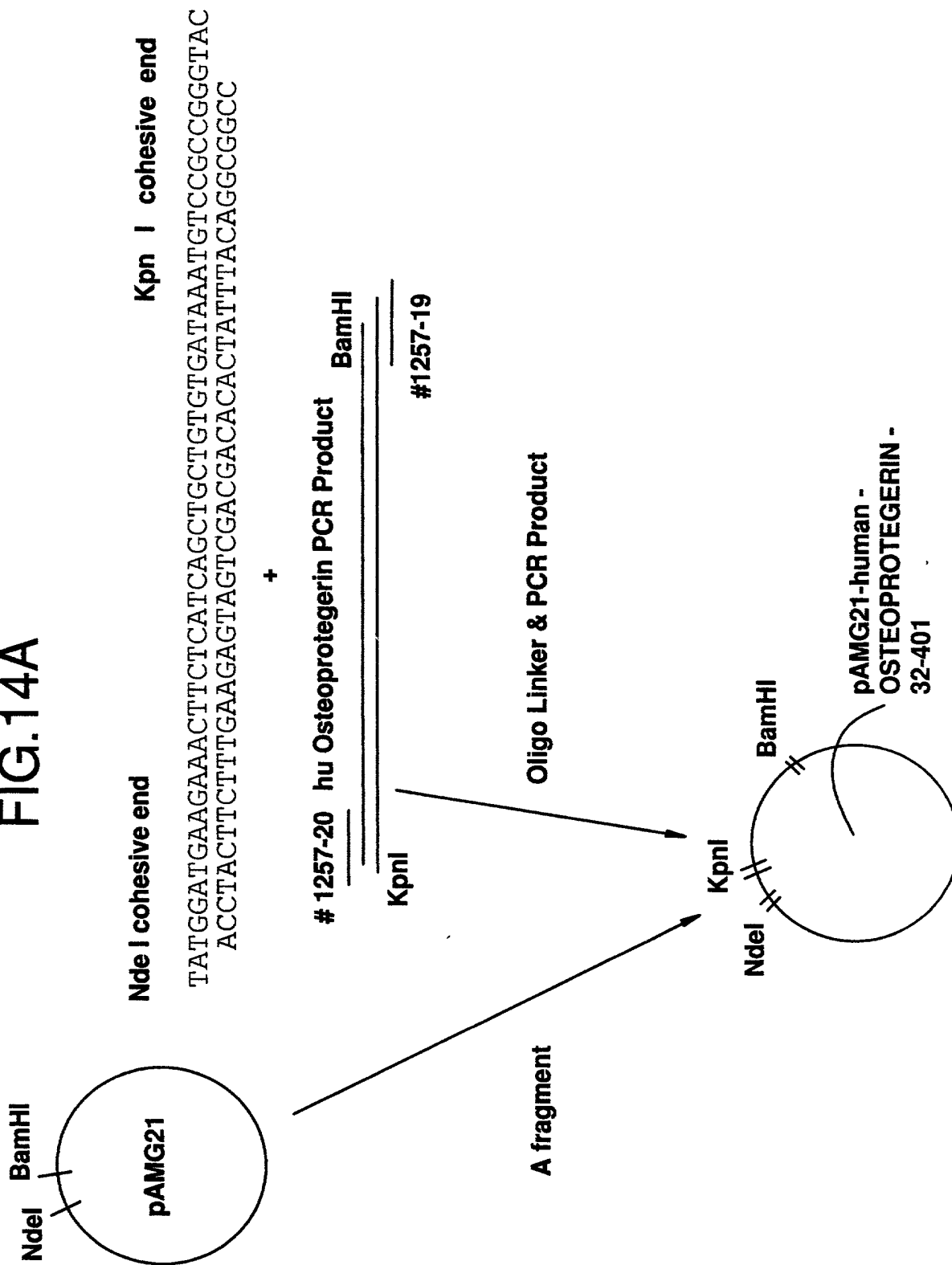


FIG.14B

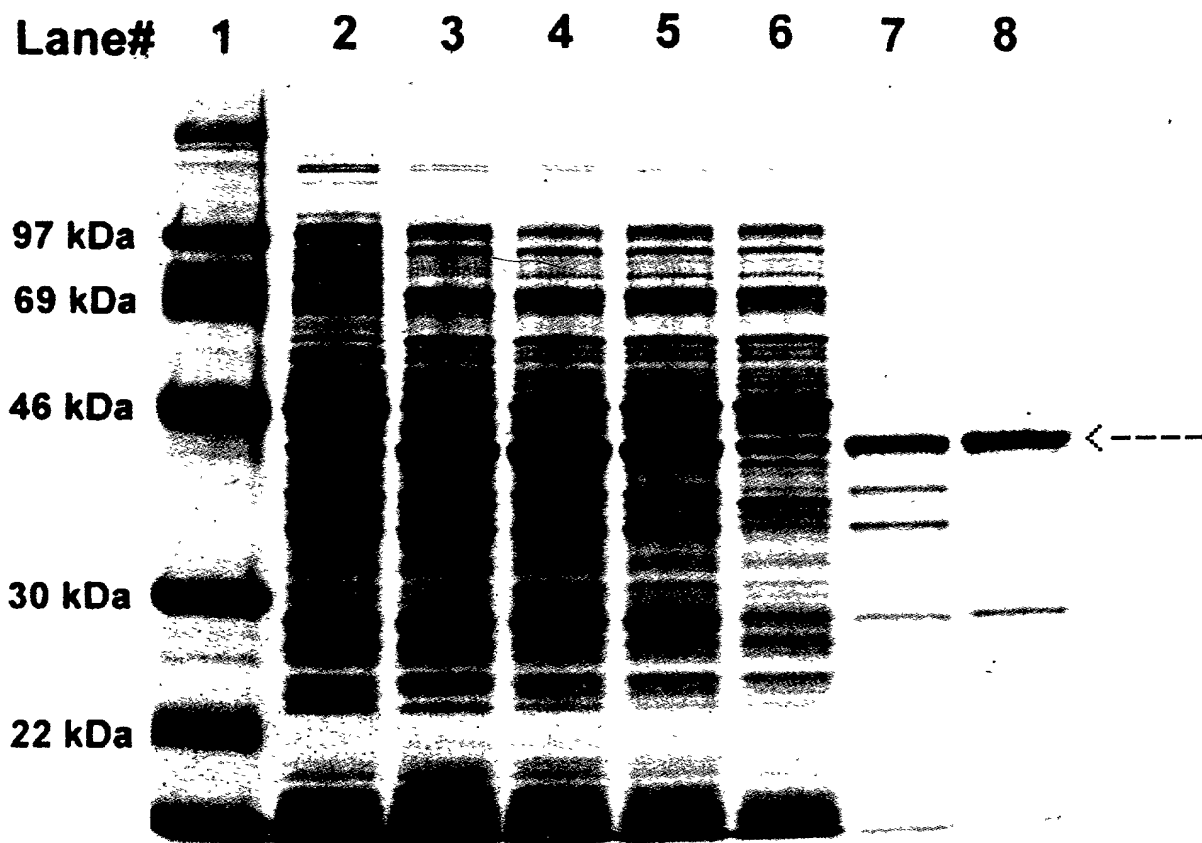


FIG. 15

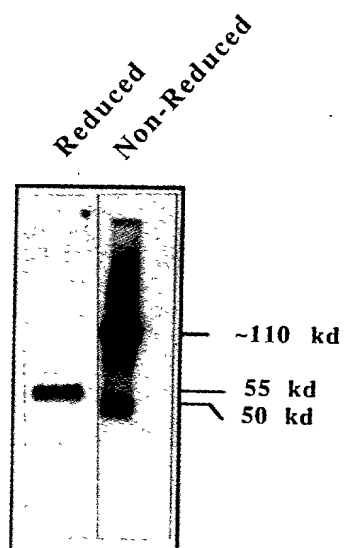


FIG.16A

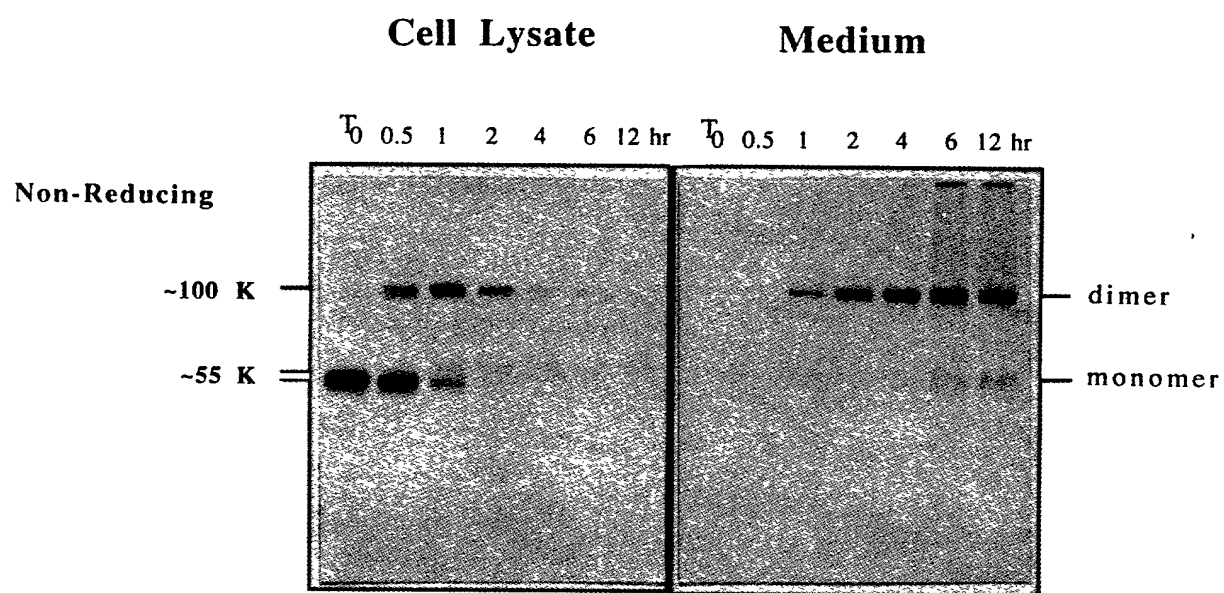


FIG.16B

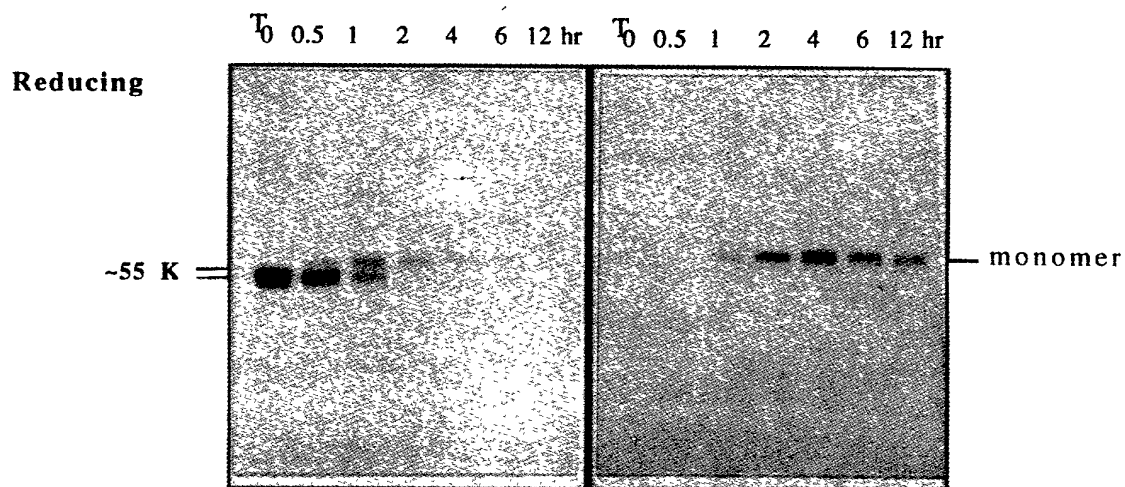
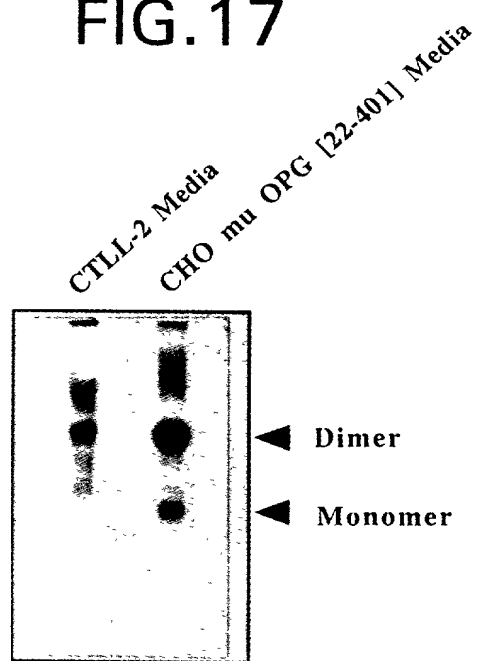


FIG.17



# FIG.18

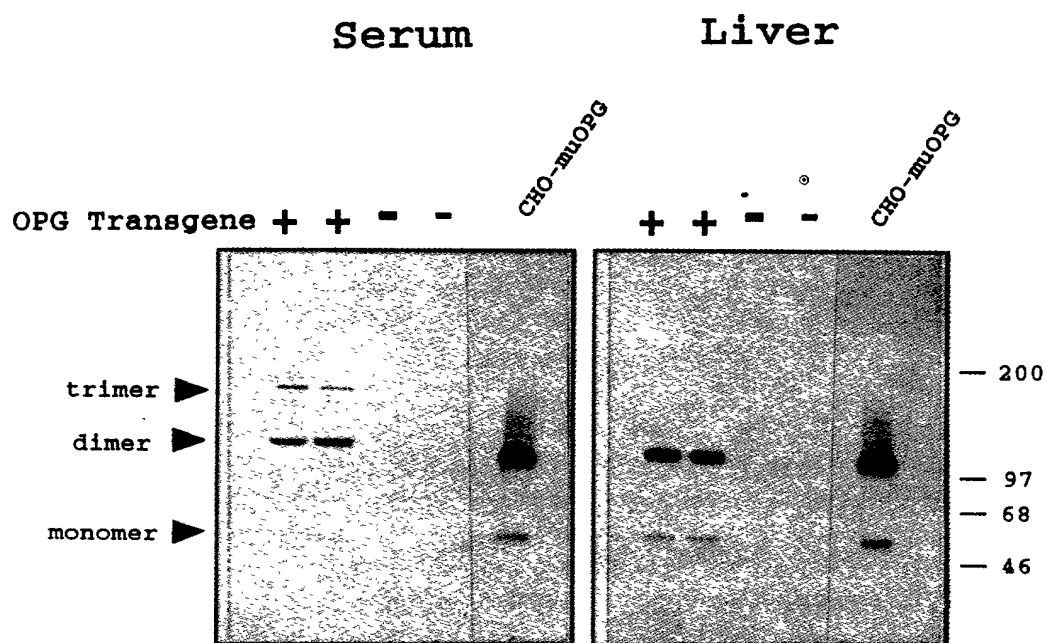


FIG.19A

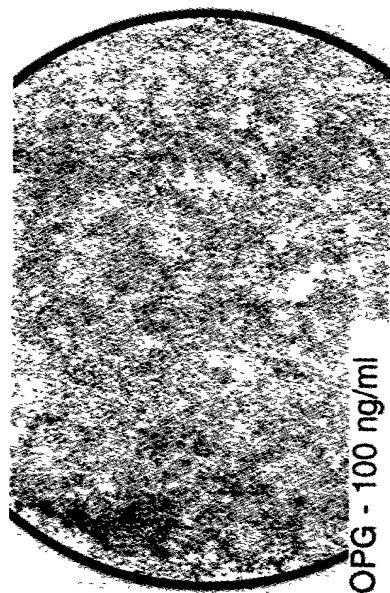


FIG.19B

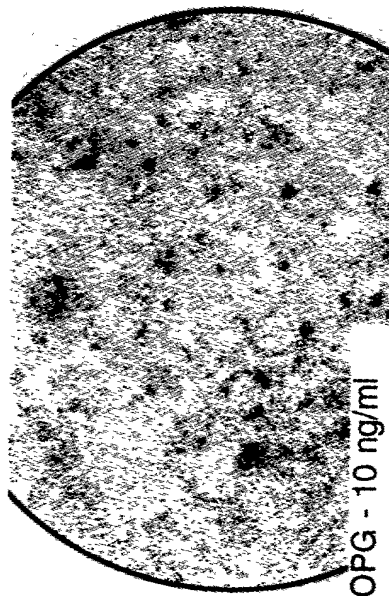


FIG.19C

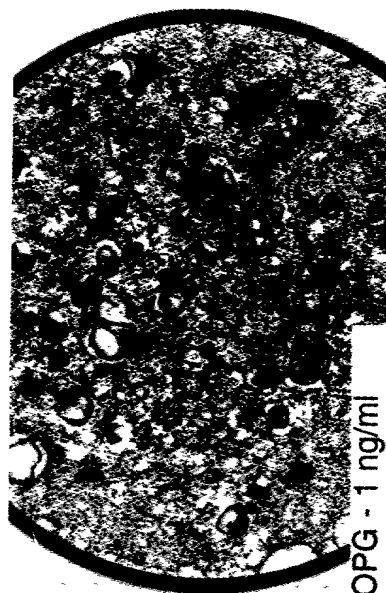


FIG.19D

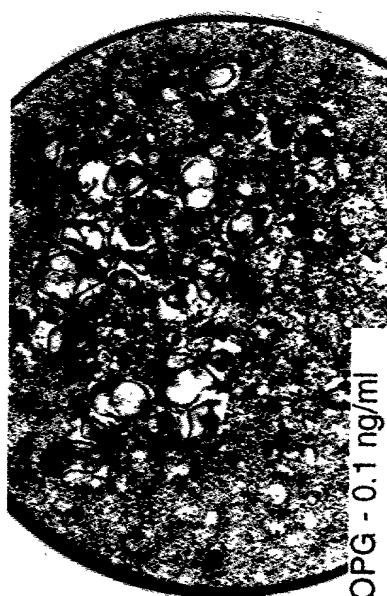




FIG.19E

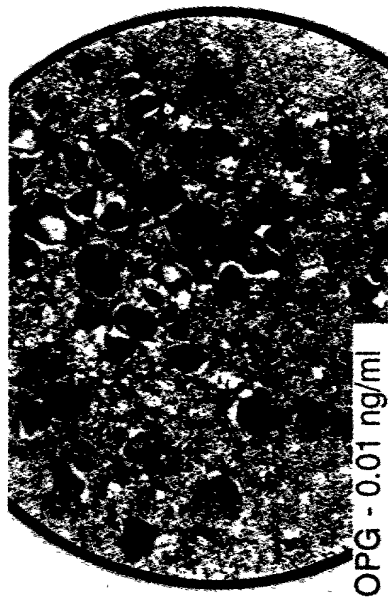


FIG.19F

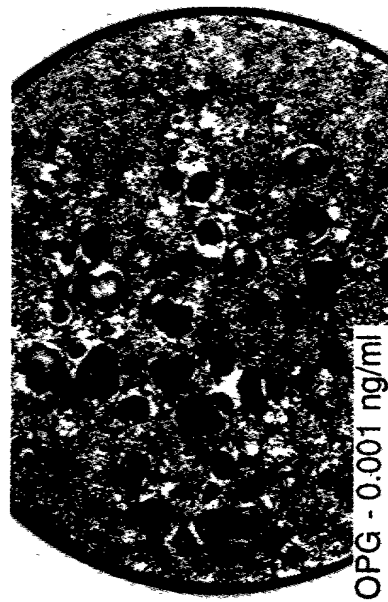


FIG.19G

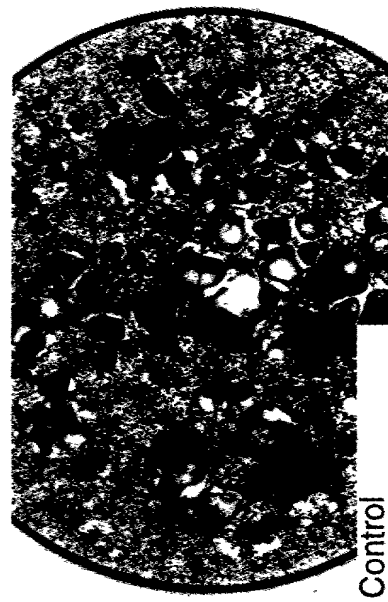
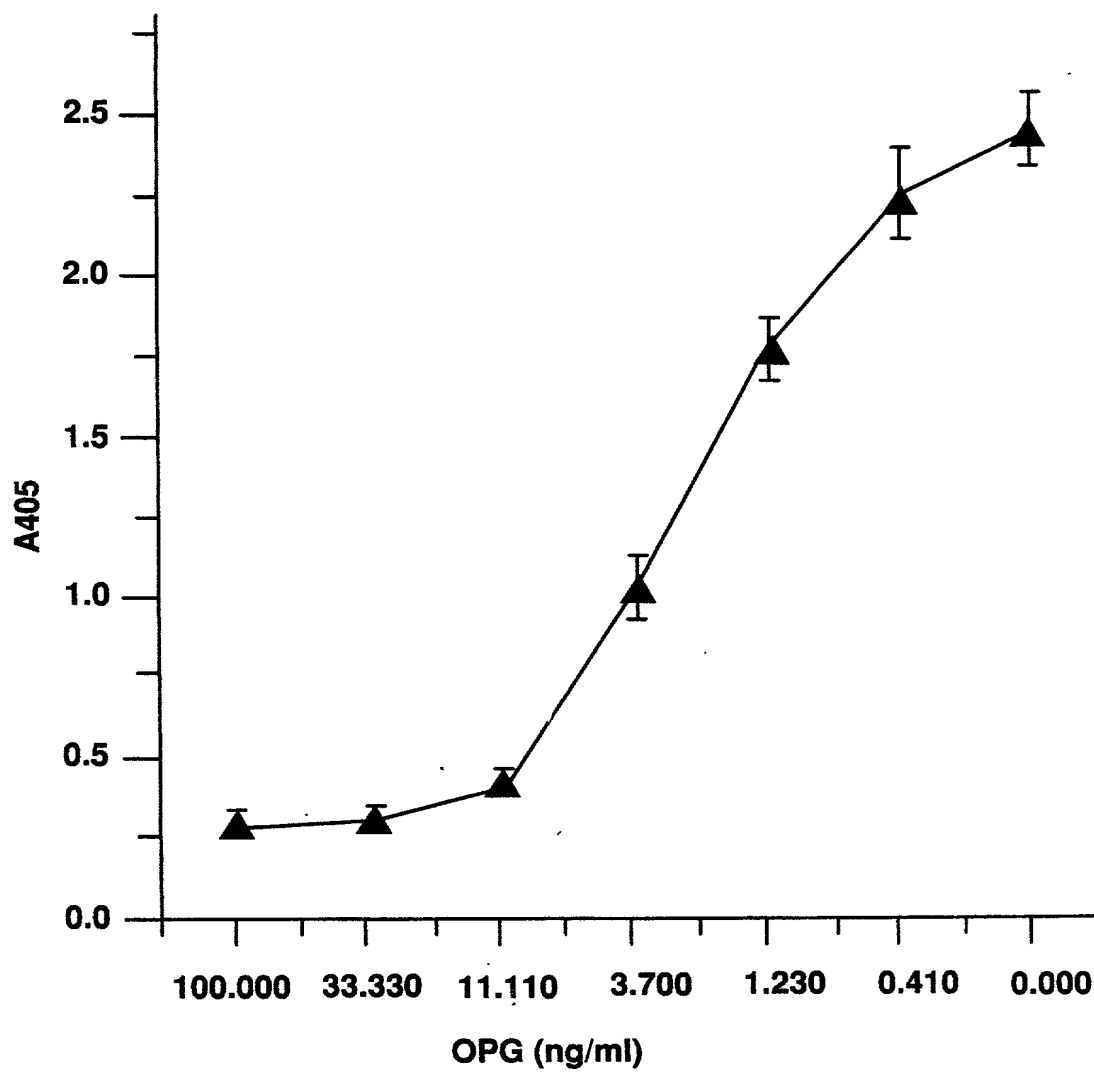
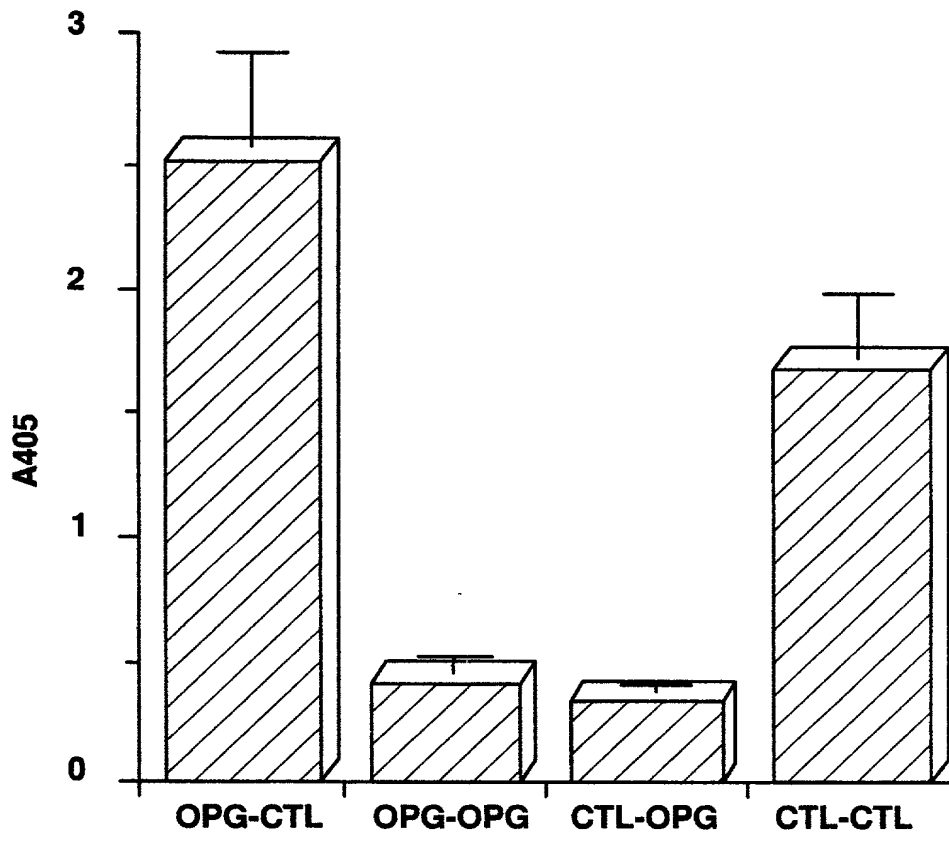


FIG.20



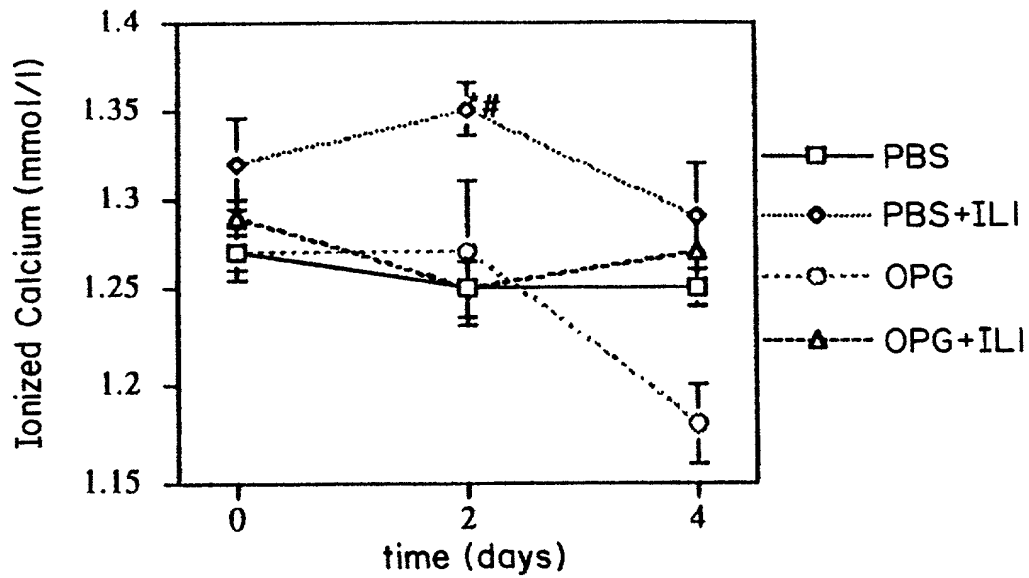
# FIG.21



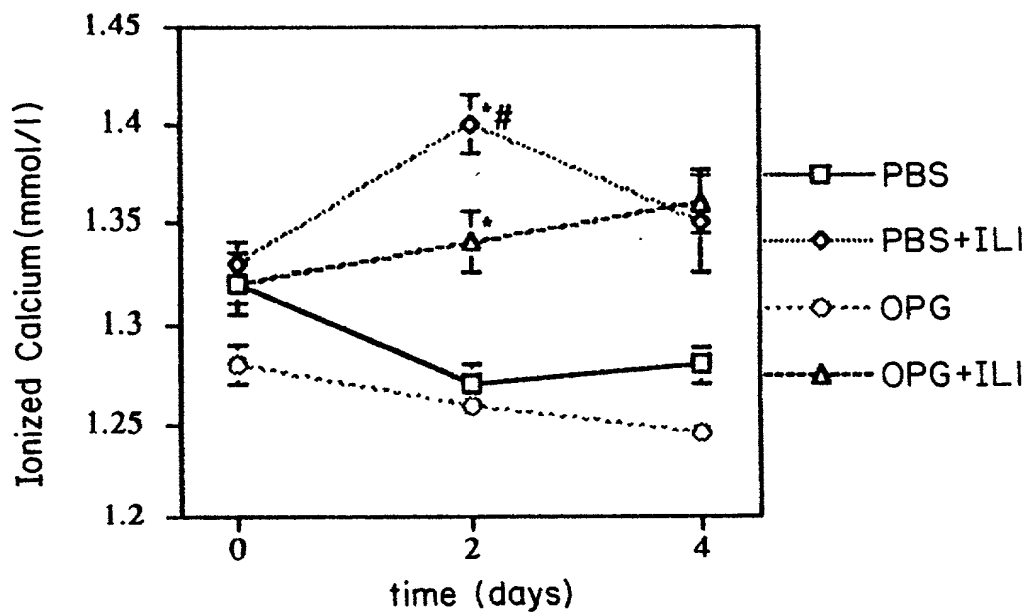
## Legend

Growth Bone marrow cells CSF -1		Intermediate PGE2 + CSF-1		Terminal ST2 cells 1,25 (OH)2 D3 Dexamethasone	
4 days		2 days		8 - 10 days	
Groups		OPG		OPG	
CTL - CTL		---		---	
OPG - CTL		100 ng/ml		---	
OPG - OPG		---		100 ng/ml	
OPG - OPG		100 ng/ml		100 ng/ml	

# FIG.22A



# FIG.22B



\* Different to PBS,  $p < 0.05$

# Different to OPG + IL1,  $p < 0.05$

FIG.23A

PBS/PBS



FIG.23B

IL1/PBS

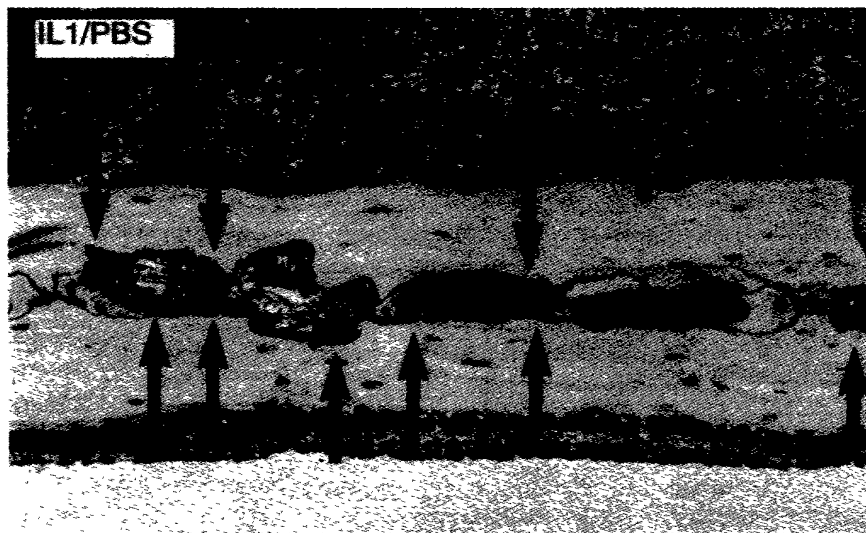


FIG.23C

PBS/OPG



FIG.23D

IL1/OPG

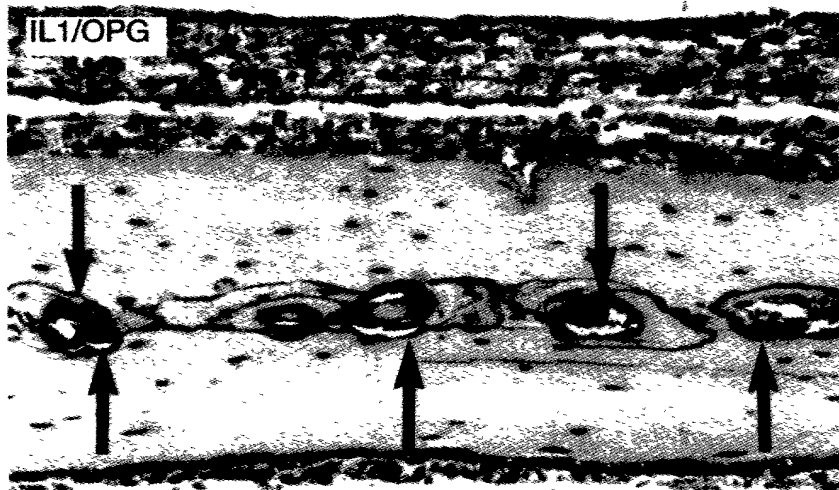


FIG. 24A

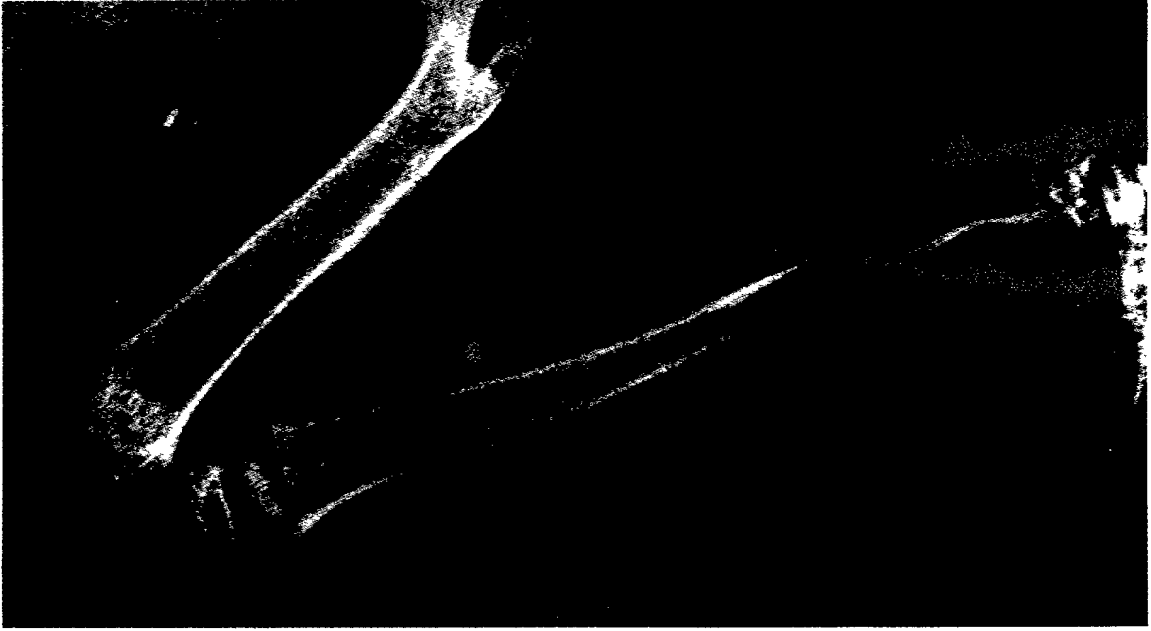
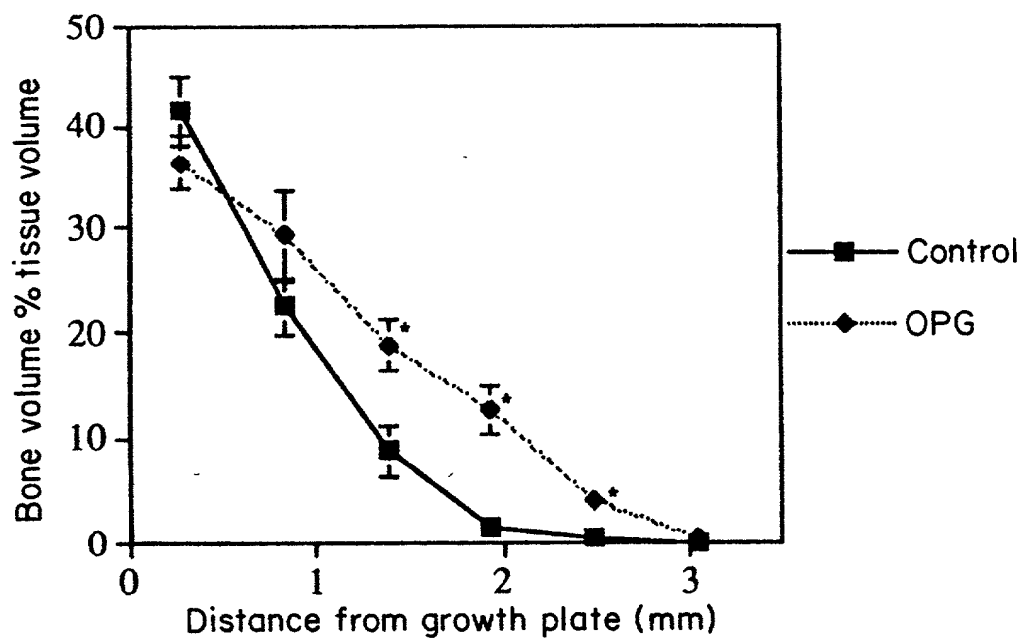


FIG. 24B



FIG.25



\* Different to control  $p < 0.01$



FIG.26A

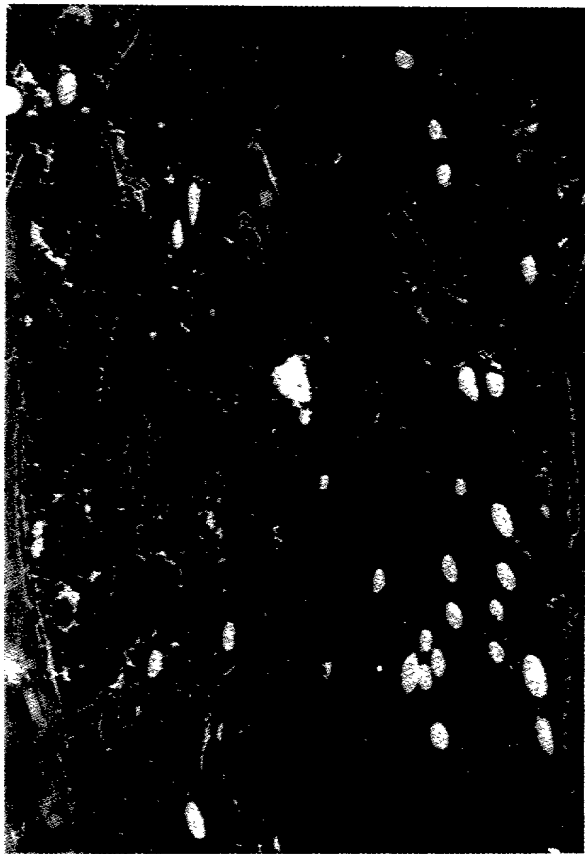


FIG.26.B



FIG.27

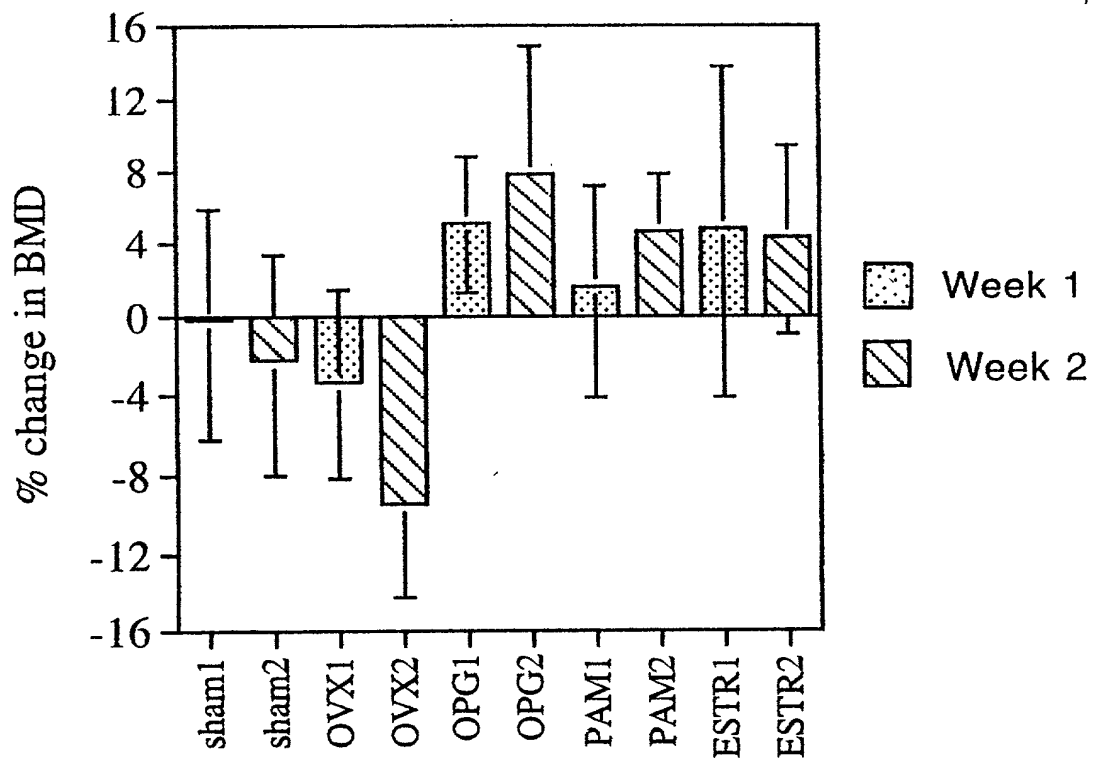
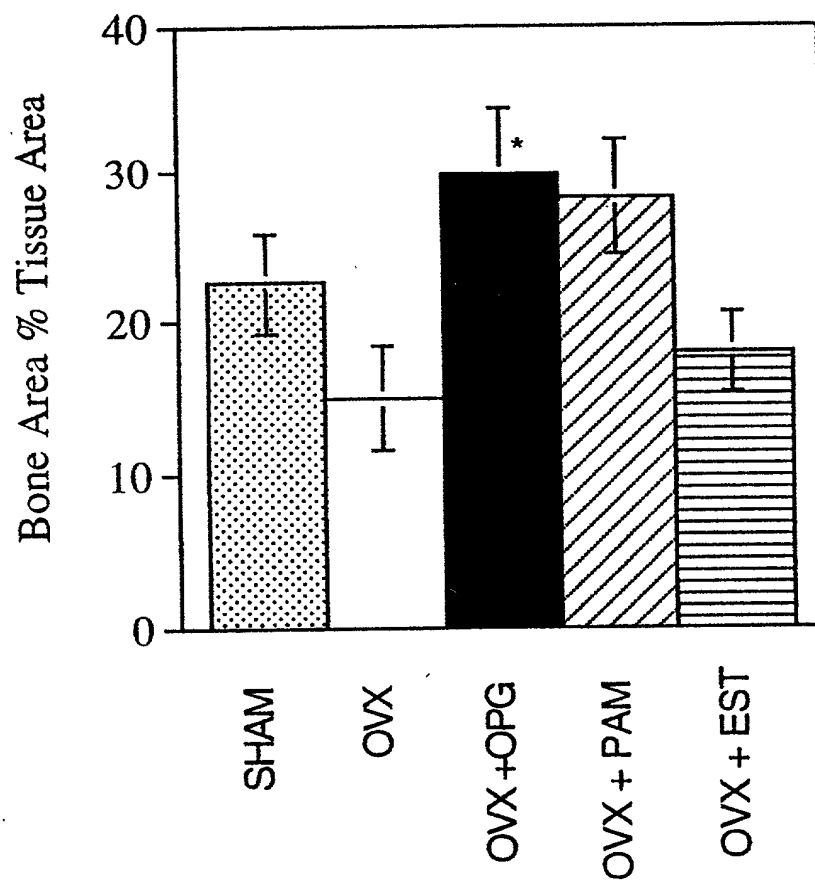


FIG.28



\* Different to OVX  $p < 0.05$

**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed below) or a joint inventor (if plural names are listed below) of the invention entitled

**OSTEOPROTEGERIN**

which is described and claimed in the specification which:

☐ is attached hereto.

☒ was filed on December 20, 1996  
as Application Serial No. 08/771,777  
and was amended on \_\_\_\_\_ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS
08/577,788	12/22/95	Pending
08/706,945	09/03/96	Pending

**Power of Attorney:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Ron K. Levy, Registration No.: 31,539; Steven M. Odre, Registration No.: 29,094; and Robert B. Winter, Registration No.: 34,458, said attorney(s)/agent(s) to have in addition full power of revocation, including the power to revoke any power herein granted.

Please send all future correspondence to:

U.S. Patent Operations/RBW  
M/S 10-1-B  
AMGEN INC.  
Amgen Center  
1840 Dehavilland Drive  
Thousand Oaks, California 91320-1789

Direct Telephone Calls To:

Robert B. Winter  
Attorney/Agent for Applicant(s)  
Registration No.: 34,458  
Phone: (805) 447-2425  
Date: April 9, 1997

**CERTIFICATE OF MAILING**

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231, on the date appearing below.

4/11/97

Date

Robert B. Winter

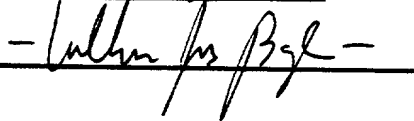
Signature

**DECLARATION AND POWER OF ATTORNEY (cont'd)**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full Name of Sole  
or First Inventor:William J. Boyle

Inventor's Signature:

Date: 4.7.97

Residence:

11679 Chestnut Ridge Street Moorpark, CA 93021 USA

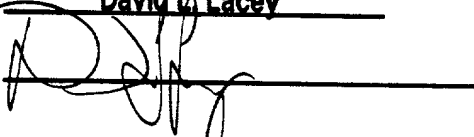
Post Office Address:

11679 Chestnut Ridge Street Moorpark, CA 93021 USA

Citizenship:

U.S.A.Full Name of Second  
Joint Inventor, if Any:David L. Lacey

Inventor's Signature:

Date: 4-8-97

Residence:

614 Paseo Vista, Thousand Oaks, CA 91320

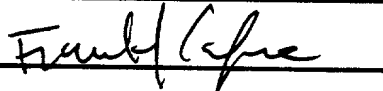
Post Office Address:

614 Paseo Vista, Thousand Oaks, CA 91320

Citizenship:

U.S.A.Full Name of Third  
Joint Inventor, if Any:Frank J. Calzone

Inventor's Signature:

Date: 4-8-97

Residence:

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Post Office Address:

841 Pine Crest Circle, Westlake Village, CA 91361

Citizenship:

U.S.A.

Docket No.: A-378-CIP2

Full Name of Fourth  
Joint Inventor, if Any: Ming-Shi Chang

Inventor's Signature: *Ming-Shi Chang*

Date: 4/9/97

Residence: 736 Calle Las Colinas, Newbury Park, CA 91320

Post Office Address: 736 Calle Las Colinas, Newbury Park, CA 91320

Citizenship: U.S.A.

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant(s): Boyle et al.  
Serial No.: Continuation of 09/132,985  
Filed: September 24, 1999  
For: Osteoprotegerin  
Docket No.: A-378-CIP2C2

**ATTORNEY'S STATEMENT PURSUANT TO § 1.821(e)**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

The computer readable form in this continuation application, is identical with that filed in application Serial No. 09/132,985, filed on August 12, 1998. In accordance with 37 CFR § 1.821(e), please use the only computer readable form filed in that application as the computer readable form for this application. It is understood that the Patent and Trademark Office will make the necessary change in the application number and filing date for the computer readable form that will be used for this application. A paper copy of the Sequence Listing is provided herewith. I hereby state that the paper copy and the computer readable form (CRF) of the "Sequence Listing" for the above-mentioned patent application are the same.

Respectfully submitted,

Robert B. Winter  
Attorney/Agent for Applicant(s)  
Registration No.: 34,458  
Phone: (805) 447-2425  
Date: September 24, 1999

Please send all future correspondence to:

U.S. Patent Operations/RBW  
Dept. 430, M/S 27-4-A  
AMGEN INC.  
One Amgen Center Drive  
Thousand Oaks, California 91320-1789

**EXPRESS MAIL CERTIFICATE**

"Express Mail" mail labeling number: EL198792556US

Date of Deposit: September 24, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to Boy Patent Application, Assistant Commissioner for Patents, Washington, D.C. 20231

R. Craft  
Printed Name

J. Craft  
Signature